MAMMALIAN NEURALIZED FAMILY TRANSCRIPTIONAL REGULATORS AND USES THEREFOR

Background of the Invention

Field of the Invention

The described invention relates to the family of mammalian Neuralized (*neu*) genes, proteins encoded by those genes (Neu), expression patterns of the gene family, their function as a transcriptional regulator, and the proteins with which the Neu family of proteins interacts. Additionally, therapeutic and diagnostic uses for the Neu family of proteins and agents that bind thereto are also provided.

Description of the Related Art

Development and functioning of the nervous system requires orchestrated action of thousands of transcriptional regulators. Balance between transcriptional activators and repressors determines the spectrum of expressed genes. The molecular basis of the initial stages of neurogenesis as well as several aspects of neuronal differentiation have been extensively studied. As a result, a variety of transcriptional activators and repressors have been discovered and characterized. Our understanding of these systems and their component interactions, however, is far from complete. Little is known about the molecular mechanisms that support neuronal circuits in developing and mature nervous systems or the molecular mechanisms that coordinate maintenance of the differentiated state.

The process of lateral inhibition prevents neighboring cells from developing into the same type of differentiated cells in flies and in vertebrates. Tanabe & Jessell, *Science*, 274:1115-23 (1996). In *Drosophila*, a group of mutations has been described that shows severe defects in the process of lateral inhibition in the developing nervous system. These neurogenic mutations result in hyperplasia of the neural tissue at the expense of epidermal structures. Campos-Ortega & Jan, *Annu Rev Neurosci*, 14:399-420 (1991). The temporal and spatial expression patterns of the *neu* are compatible with its function as a neurogenic gene in *Drosophila*. The Neu protein is expressed throughout the ectoderm at the time when cell fate is determined and its expression

proceeds in neuroblasts. Boulianne, et al., *EMBO J*, 10:2975-2983 (1991). *neu* expression has been detected in actively proliferating neuroblasts in several regions of the CNS and PNS. Expression of *neu* in imaginal disc suggests that it is also involved in later stages of development. The *neu* gene encodes a RING finger (C3HC4) type zinc finger protein. The molecular function of the *Drosophila* Neu protein is unknown. Interestingly, it was discovered that EST data bases contain a homologue of Neu suggesting that a family of Neu-like proteins is present in *Drosophila*.

Current studies of the brain development in *Drosophila* and vertebrates, indicate that many basic molecular and genetic mechanisms involved in neurogenesis are highly conserved. During development of the nervous system, neural cell specification is acquired through the series of progressive restrictive steps. In *Drosophila*, neural precursors are first specified by proneural genes including basic helix-loop-helix (bHLH) transcription factors of *atonal* and *achaete-scute* complex. Simpson, *Neuron*, 15:739-742 (1995). The process of lateral inhibition, which further restricts the developmental potential of neuroectodermal cells is regulated by neurogenic genes such as *Notch, mastermind, big brain, Delta, Enhancer of split,* and *neuralized.* Analysis of the function of these neurogenic loci in the *Drosophila* embryo has revealed that mutations in any of these genes result in hyperplasia of neural tissue at the expense of epidermal structures and also cause defects in tissues derived from mesoderm and endoderm. Campos-Ortega and Jan, *Annu. Rev Neurosci*, 14:399-420 (1991); Harentstein et al., *Development*, 116:1203-1220 (1992). Vertebrate homologues of *Notch, Delta* and the proneural/neurogenic genes of *atonal, achaete-scute, hairy,* and *Enhancer of Split* complex have been identified and recent work, mostly in Xenopus and mouse, suggests that their role in neurogenesis is conserved. Lewis, *Curr Opin Neurobiol*, 6:3-10 (1996); Kageyama et al., *Int J Biochem Cell Biol*, 29:1389-1399 (1997); and Beatus, Lendahl, *J Neurosci Res* 54:125-136 (1998). For example, postnatal Notch signaling affects the elaboration of different body systems and regulates plasticity of cortical postmitotic neurons. Artavanis-Tsakonas et al., *Science*, 284:770-776 (1999); Redmond et al., *Nat Neurosci*, 3:30-40 (2000); and Sestan et al., *Science*, 286:741-746 (1999).

The last few years have brought the identification and characterization of many new key regulators of vertebrate neurogenesis. Recently, a human homologue of *Drosophila* Neu gene was isolated and its expression in the adult nervous system and in tumors of neuroectodermal origin, such as astrocytomas, was characterized. Nakamura, et al., *Oncogene* 16(8):1009-1019 (1998). Nakamura and others (1998) hypothesized that *h-neu1* plays a role in determination of cell fate in the central nervous system and may act as a tumor suppressor which inactivation could be associated with malignant progression of astrocytic tumors. A homology search in human, rat, and mouse EST databases revealed three new mammalian Neu homologs, suggesting that a family of Neu-like proteins exists in mammals.

Summary of the Invention

The disclosure relates to isolated polynucleotides and purified polypeptides of the Neu family of proteins, which have been shown to demonstrate transcriptional regulatory activity. For example, the purified polynucleotide can encode a Neu polypeptide, wherein the Neu polypeptide comprises at least one neuralized homology repeat domain and a C3HC4 RING-zinc finger domain is disclosed. A purified Neu polypeptide, wherein the Neu polypeptide comprises at least one neuralized homology repeat domain and a C3HC4 RING-zinc finger domain is disclosed. Antibodies capable of specifically binding to the disclosed Neu polypeptides are disclosed. Vectors expressing the disclosed Neu protein coding regions and host cells containing the vectors are disclosed. Methods of making the Neu proteins disclosed are also provided, as are methods of identifying binding partners that interact with a Neu protein family member.

Brief Description of the Drawings

Figure 1 shows the primary structure of neu1 protein isoforms in mouse and rat.

Figure 2 is an analysis of *neu1* mRNA expression by RNase protection assay in mouse and rat.

Figure 3 is an in situ hybridization analysis of *neu1* mRNA expression in the developing and adult mouse brain.

Figure 4 is an in situ hybridization analysis of *neu1* mRNA expression in adult rat brain.

Figure 5 shows expression of *neu1* mRNA in an adult rat nervous system.

Figure 6 shows cellular localization of *neu1* mRNA in an adult rat nervous system.

Figure 7 shows the combined analyses of *in situ* hybridization of *neu1* mRNA and immunohistochemistry of the neuronal marker neuN.

Figure 8 shows *neu1* mRNA expression in the hippocampus of adult rat brain after kainic acid treatment.

Figure 9 shows transcriptional analysis from various promoters in transient expression assays.

Figure 10 shows that neuralized homology repeat domains of neu1 mediate the transcriptional repression when fused to the DNA binding domain of Gal4.

Figure 11 shows the subcellular localization of neu-FLAG and neu-EGFP fusion proteins in Neuro2A cells, using FLAG immunofluorescence using anti-FLAG antibody *(A, E, F, G)* and direct fluorescence of EGFP fusion proteins *(B, C, D, H)*.

Figure 12 shows alignment of neuralized homology repeat domains of human Neu1, Neu2, and Neu3 proteins.

Figure 13 shows the analysis of *neu2* mRNA expression by RNase protection assay in mouse and rat.

Figure 14 shows the analysis of *neu3* mRNA expression by RNase protection in mouse.

Figure 15 is an analysis of the expression of rat Neu1 interactors by RNase protection assay.

Detailed Description of the Preferred Embodiments

The invention described herein relates to the identification of members of the Neuralized (*neu*) family of genes and their respective proteins, and aspects of their structure and function as transcriptional regulators. The invention relates to the characteristics of mammalian *neu* gene family's conserved structural motifs, related function as transcriptional regulators, and functional features of the family relating to

expression patterns and potential interactions with interaction-partners, providing tools with which to explore the Neu-related regulatory cascades that operate in neurons and other cell types.

Structure

The invention described herein relates to the neuralized (Neu) family of transcriptional regulators that contain a C-terminal C3HC4 RING zinc finger and at least one neuralized homology repeat domain. The term "neuralized" (or "Neu"), as used herein, includes all members of the Neu family such as Neu1, Neu2, Neu3, Neu4 coding sequences and proteins. These proteins belong to a family of proteins that share C-terminal C3HC4 RING zinc finger and at least one neuralized homology repeat domain. Neuralized homology repeat domains represent a novel class of transcription repression domains that regulate transcription of a large number of genes.

The C3HC4 RING-zinc finger motif is a cystine-rich amino acid sequence motif found in the sequence of the human RING gene. Freemont, et al., *Biochem J*, 278:1-23 (1991); Freemont, *Ann NY Acad Sci*, 684:174-192 (1993). The motif can be described as $C\text{-}X_2\text{-}C\text{-}X_{(9\text{-}27)}\text{-}C\text{-}X_{(1\text{-}3)}\text{-}H\text{-}X_2\text{-}C\text{-}X_2\text{-}C\text{-}X_{(4\text{-}48)}\text{-}C\text{-}X_2\text{-}C$, where C is a cysteine, H is a histamine, and X can be any amino acid. This family includes genes that are involved in the regulation of development, differentiation, apoptosis, oncogenesis, and membrane trafficking.

Several isolated and characterized RING-zinc finger proteins have proposed roles in gene regulation. Although the precise function of the RING-zinc finger domain is unknown, indirect data demonstrates that the RING-zinc finger domain could function as a DNA-binding or protein-protein interaction domain. These two functions are related. Several transcriptional repressors which act as dimers have been characterized. Their structures comprise both dimerization and DNA-binding surfaces. For example, well known transcriptional regulators that contain RING-zinc finger domains are BRCA1 (Miki et al., *Science*, 266(5182):66-71 (1994)) and MEL18 (Kanno et al., *EMBO J.* 15;14(22):5672-8 (1995)), a polycomb group-related transcriptional regulator.

Function and Interactions

Neu function as a transcriptional regulator

Mammalian Neu1 acts as a powerful transcriptional repressor in transient expression assays and silences both, TATA-containing and TATA-less promoters, including the promoters of NGF, BDNF, NF-L, and GAP-43. NHRs function as transcription repression domains, suggesting that NHR-containing proteins represent a novel class of transcriptional repressors. It is likely that mammalian Neu1 mediates transcriptional repression through protein-protein interactions. Like several known repressors, mammalian Neu1 could function through interaction with co-repressors such as dCtBP or mammalian homologues of Groucho, and general repressor complexes, such as NC2, Mot1, or Not to interfere with the function of Pol II complex. Maldonado et al., *Cell*, 99:455-458 (1999) and Mannervik et al., *Science*, 284:606-609 (1999). Alternatively, repression could be achieved through chromatin remodeling by recruiting the histone deacetylase complexes (HDACs). Glass and Rosenfeld, *Endocr Rev*, 21:447 (2000); Knoepfler and Eisenman, *Cell*, 99:447-450 (1999); and Torchia et al., *Curr Opin Cell Biol*, 10:373-383 (1998). Current data suggest that the mechanism of Neu1 repression does not include a HDAC complex in neuroblastoma Neuro2A cells, as the HDAC inhibitor trichostatinA did not relieve m-Neu1-mediated repression in these cells.

Neu is a shuttling protein with dominant cytoplasmic localization as a result of a nuclear import combined with an efficient export

A putative nuclear localization signal has been identified in the N-terminus of d-Neu, however, the NLS sequence identified in d-Neu, is not conserved in mouse, rat and human Neu proteins. Boulianne et al., *EMBO J* 10:2975-2983 (1991) and Price et al., *EMBO J*, 12:2411-2418 (1993). The weak NLS sequences (HKAVKR (SEQ ID NO: 43), RLKITKK (SEQ ID NO: 44)), that were identified in mammalian Neu1 proteins, have been suggested to regulate nuclear import of a fraction of the synthesized protein. Boulikas, *J Cell Biochem*, 60:61-82 (1996). Indeed, m-Neu1 resides both in the cytoplasm and in the nucleus, revealing that it is the subject of regulated nuclear import. Recent studies have shown that importin-α family members are involved in the

formation of the NLS receptor complexes that govern the protein transport to the nucleus. Ullman et al., *Cell*, 90:967-970 (1997); and Izaurralde and Adam, *RNA*, 4:351-364 (1998). Interestingly, importin-α3 was identified here as one of the m-Neu interacting proteins by yeast two-hybrid screening. Furthermore, the results discussed herein also demonstrate that the CRM1/exportin1-related export pathway controls the nucleocytoplasmic shuttling of Neu1, since the nuclear export of a tagged-m-Neu1 fusion protein is blocked by LMB. LMB is a microbial metabolite that inactivates the nuclear export by interfering with the binding of CRM1/exportin1 to the nuclear export signals. Fornerod et al., *Cell*, 90:1051-1060 (1997); Kudo et al., *Exp Cell Res*, 242:540-547 (1998); Kudo et al., *Proc Natl Acad Sci USA*, 96:9112-9117 (1999); Fukuda et al., *Nature*, 390:308-311 (1997); and Nishi et al., *J Biol Chem*, 269:6320-6324 (1994). These data reveal that mammalian Neu1 function is additionally regulated by nucleocytoplasmic shuttling.

Neu function as a calcium-signal transducer

Transcriptional activity of Neu is controlled by calcium (Ca^{2+}) signaling that regulates Neu translocation into the nucleus where Neu acts as a transcriptional repressor. It has been long known that intracellular calcium controls a variety of brain- and muscle cell functions. Since Ca^{2+} levels regulate nucleocytoplasmic shuttling of transcription factors (Crabtree, *Cell*, 96:611-614 (1999)), Neu proteins could function as mediators of calcium signaling to the nucleus to regulate gene expression. There is evidence that in *Drosophila* and also in vertebrates, ligand activated Notch is subjected to proteolytic cleavage and transported to the nucleus where it acts as a transcriptional regulator (Jarriault et al 1995; Ohtsuka et al., 1999). Interestingly, recently it was shown that d-neu protein is associated primarily with the plasma membrane (Yeh et al., 2000). This, however, does not exclude the possibility that Neu1, like Notch, may be localized also within the nucleus. Based on current knowledge it is hypothesized that Neu1 functions as a mediator of an extracellular signal from the plasma membrane to the nucleus to regulate gene expression. According to one possible scenario, Neu1 is posttranslationally modified in a signal (Ca^{2+}) -dependent fashion and subsequently translocated into the nucleus, where it functions as a transcriptional regulator.

Neurogenic function of Neu.

Drosophila neuralized (*d-neu*) and *h-neu*1 genes encode homologous (~40%) proteins with a C-terminal C3HC4 RING zinc finger domain (RZD) and one or two neuralized homology repeat (NHR) domains. Boulianne et al., *EMBO J*, 10:2975-2983 (1991) and Price et al., *EMBO J*, 12:2411-2418 (1993). *d-neu* is expressed in the ectoderm at the time when cell fate is determined, implying the role of Neu in neurogenesis. Boulianne et al., *EMBO J*, 10:2975-2983 (1991).

In *Drosophila*, *d-neu* expression has been detected earliest in the ectoderm continuing later in neuroblasts. Boulianne et al., *EMBO J*, 10:2975-2983 (1991). Since developing mouse CNS can be divided into regions that express either high or low levels of *neu1*, and as *neu1* is not expressed in proliferating regions of the nervous system, it suggests that other *neu*-related genes function in a fashion complementary to *neu1*. Indeed, based on the recent findings we could argue that *neu1* defines a new gene and protein family consisting of at least two *Drosophila* and four mammalian genes.*neu2*, second member of mammalian *neu* family of genes, is expressed at high levels in the embryonic brain, whereas the expression levels decrease during postnatal development. Recent studies of the brain development in *Drosophila* and vertebrates, indicate that many basic molecular and genetic mechanisms involved in neurogenesis are highly conserved. Lewis, *J Curr Opin Neurobiol*, 6:3-10 (1996); Kageyama et al., *Int J Biochem Cell Biol*, 29:1389-1399 (1997); and Beatus & Lendahl, *J Neurosci Res*, 54:125-136 (1998). For example, postnatal Notch signaling affects the elaboration of different body systems and regulates plasticity of cortical postmitotic neurons. Artavanis-Tsakonas et al., *Science*, 284:770-776 (1999); Redmond et al., *Nat Neurosci*, 3:30-40 (2000); and Sestan et al., *Science*, 741-746 (1999).

Earlier genetic studies in *Drosophila* suggested that *delta*, *mastermind*, *big brain*, and *neuralized* refine a signal upstream of *notch* and that *mastermind* functions upstream of all the other neurogenic genes. Campos-Ortega and Jan, *Annu Rev Neurosci*, 14:399-420 (1991); and Lieber, *Genes Dev*, 7:1949-1965 (1993). Therefore, if the function of the mammalian Neu family of proteins is conserved in conjunction with other neurogenic factors, mammalian *neu* genes refine the signal upstream of

mammalian homologues of *notch* and downstream of *mastermind* in tissues where *neu* genes are expressed, particularly in developing nervous system. Given that mammalian *neu* mRNAs encode different protein isoforms, complex regulatory circuits implicating various Neu family members are expected in different tissues. The transcription repression activities of mammalian Neu could well-explain its function as a neurogenic gene.

Neu role in cell signalling and synaptogenesis

Suppression of gene expression plays an important role in the maintenance and stability of a mature nervous system. It is essential to suppress neurite growth and extensive formation of new axons and dendrites in the adult functional nervous system and to maintain neurons in their differentiated state. Transcriptional repressors are involved and play a crucial role in the silencing of the neurite growth program.

Mammalian *neu1* shows most prominent expression in the postnatal central nervous system, revealing its function in postnatal development. *neu1* mRNA expression levels increase significantly during the early postnatal development when active synaptogenesis takes place, to reach the peak levels in the adult animal. In the adult mammalian CNS, the highest expression levels of *neu1* assign to the neurons of hippocampus, cerebral cortex, striatum, and amygdala. Whereas several brain regions such as thalamus/hypothalamus, midbrain, medulla, and also the spinal cord exhibit low expression. This indicates to the independent regulation of *neu1* expression in various brain regions by specific signaling mechanisms as well as to the requirement of Neu function for different cell-cell signaling systems.

neu1 mRNA expression studies in adult rat brain revealed that in several brain regions, particularly in the granular cells of dentate gyrus of hippocampus, *neu1* mRNA is localized in the dendrites, suggesting that synthesis of Neu1 protein also occurs in dendrites. Several data indicate that proteins locally translated from dendritic mRNAs at activated synapses provide basis for activity-dependent regulation of synaptic modulation (reviewed in Steward et al., *Neuron,* 21:741-51 (1997); Kuhl and Skehel, *Curr Opin Neurobiol*, 8(5):600-6 (1998); Schuman, *Neuron*, 23:645-8 (1999); Tiedge et al., *Science*, 283:186-7 (1999); Kiebler and DesGroseillers, *Neuron*, 25:19-28 (2000)).

Accordingly, Neu could be involved in the regulation of neuritogenesis and/or synaptogenesis, affecting the generation of the precise pattern of neuronal connectivity. Recent molecular perturbation experiments suggested that Notch1 signalling in cortical neurons promotes dendritic branching and inhibits neurite growth (Redmond et al., 2000; Sestan et al., 1999). Suggesting that the function of *neu1* to refine a signal upstream of *Notch* in *Drosophila* is evolutionary conserved in mammalian nervous system, it is possible, that Neu and Notch pathways act in an interrelated manner to confer developmental plasticity to adult neurons. The possible similarities in the molecular mechanisms of function of two genes, *Notch* and *neu1*, that were first discovered as neurogenic, are appealingly apparent.

Neu role related to repair and regeneration after injury to the CNS

A specific temporal order of events at the cellular and molecular level occurs in response to injury to the brain. Injury-compromised neurons degenerate while surviving neurons undergo neuritogenesis and synaptogenesis to establish neuronal connectivity destroyed in the injury. In the brain, after kainate-induced change in neuronal activity (a neurotoxic, excitotoxic or ischemic insult), it was observed that a consistent down-regulation of *neu1* mRNA in the hippocampal formation with a strong reduction in the molecular layer where granule cell dendrites were present. It is suggested, that after injury of the CNS, down-regulation of *neu1* mRNA expression leads to reduced levels of Neu1 protein that is essential for derepression of the transcription of its target genes related to repair and regeneration, such as growth factors and synaptic proteins.

Neu role in memory and learning

Another function of the Neu family of transcription factors is mediation of Ca^{2+} signaling in a variety of neural processes including learning and memory. A member of Homer family of proteins, Homer2a, is referred to as an interactor of human Neu1 (GenBank Acc. No. AF081530). Homer proteins are enriched in excitatory synapses, bind group I metabotropic glutamate receptors (mGluR), and NMDA receptor interacting Shank proteins and thus can link NMDA and group I mGluR signaling pathways (reviewed in Xiao et al., Curr Opin Neurobiol, 10:370-74 (2000)). Given that

Homer2a and Neu1 are co-expressed in various brain structures, it is highly conceivable that Neu1 participates in the regulation of glutamate receptor signalling in the adult brain. Glutamate receptor signaling has been implicated in several forms of activity-dependent synaptic plasticity, neurodegenerative diseases, cortical development and addiction. Consequently, if Neu is involved in glutamate receptor signaling, then the change in Neu structure, expression or function could lead to various developmental disorders and mental diseases.

Thus, it is highly conceivable that *neu1* mRNA expression is regulated by physiological neuronal synaptic activity leading to reduced levels of Neu1 protein and derepression of the transcription of its target genes in the processes involving memory and learning. This function of the Neu family transcription factors makes them a good target for a variety of drugs that control different processes in the brain, during development and in disease. Manipulating this function of Neu can be used to control a variety of diseases including depression, pain, anxiety, and neurodegenerative diseases.

Neu role in tumorigenesis

Also, this invention relates to the role of the Neu family of factors in the development of tumors, since the human *neu*1 gene has been mapped to chromosome 10 within a region which is frequently deleted in gliomas. *neu1* is expressed at varying levels in different neural and neuroendocrine tumors, including neuroblastomas, carcinoids, non-small cell lung cancers, and gliomas, suggesting that expression of Neu family of proteins is applicable as tumor-specific markers in clinical tumor diagnostics. Since over-expression of Neu1 blocks DNA synthesis in neuroblastoma and glioma cells, Neu family of proteins may function also as tumor suppressor genes.

Neu role in myogenesis and development of other organ systems.

The described herein, especially in the Examples below, show that expression of *neu1* and *neu2* is found to be high in at late embryonic and adult stages of development, also in developing heart and testes. *neu3* was found to be widely expressed, with highest levels in immune tissues spleen and thymus and in lung. Expression of *neu4* was detected only in muscle and heart. Since Neu proteins are expressed at various levels in

many different body systems, the role of Neu family of proteins in several developmental pathways is apparent. Studies of *neu*^{mut} flies have reported the overproduction of *nautilus* (*nau*) expressing cells in embryonic and muscle defects in adult stages of development. Corbin et al., *Cell*, 67:311-323 (1991); Hartenstein et al., *Development*, 116:1203-1220 (1992). nau is a myogenic bHLH factor that plays a role in the differentiation of muscle progenitors in *Drosophila*. Keller et al., *Dev Biol*, 181:197-212 (1997); Keller et al., *Dev Biol*, 202:157-171 (1998). Notch signaling has been shown to inhibit MyoD expression and block myogenesis in mouse. Kuroda et al., *J Biol Chem,* 274:7238-7244 (1999). It is possible that during mammalian myogenesis Neu refines Notch signaling by regulating expression of *nau* mammalian homologues (MyoD, myf5, myogenin and MRF4).

Another function of the Neu family of transcription factors is mediation of Ca^{2+} signaling in muscle and other tissues. This function of the Neu family transcription factors makes them a good target for a variety of drugs that control different processes in the muscle, and in those tissues where Neu is expressed. Manipulating Neu expression and function can be used to control a variety of diseases including cancer, and muscle-degenerative diseases, dystrophinopathies, Brody's disease, and malignant hyperthermia (the last three are caused by the functional alterations of Ca(2+) signaling).

Neu interactions with neurogenic genes

Additionally, activity of transcriptional regulators is modulated through interactions with other regulatory factors. Analyses of interactions between *Drosophila* neurogenic loci has revealed that *neu* appears to act upstream of *notch* (N), *enhancer of split* (E(spl)) and *delta* (Dl), and downstream of *mastermind* (mam). Boulianne et al., EMBO J. 10(10), 2975-2983 (1991). Interaction between *neu* and *E(spl)* is observed in the dominant mutation *E(spl)Dl*, which is a mutation that enhances the phenotype of split whereas *split* is a mutation in the *N* gene. Enhancement of the *split* phenotype increases in the presence of additional copies of *neu* gene, but decreases in heterozygotes for *neu* mutations. Molecular interactions between these *Drosophila* neurogenic genes are unknown. Expression of rat SHARP1 gene (Rossner, et al., *Mol*

Cell Neurosci, 10(3-4):460-475 (1997)), one of the vertebrate homologues of *Drosophila E(spl),* is almost identical to the *neu1* expression pattern during development and in adult tissues. The similarity in expression patterns between *neu1* and SHARP1 suggests that products of these genes could reciprocally affect the function of the other as it occurs in *Drosophila* between *neu* and *E(spl)* genes.

Neu interactions with proteins implicated in nuclear transport

The nucleocytoplasmic transport of functional molecules is mediated bidirectionally through the nuclear pore complex (NPC), which spans the double membranes of the nuclear envelope. It has recently been shown that signaling between the nucleus and the cytoplasm plays a key role in coordinating the cellular processes such as the cell cycle and cell differentiation (Yoneda, Cell Struct Funct 25:205-206. 2000). The weak NLS sequences (HKAVKR (SEQ ID NO: 43), RLKITKK (SEQ ID NO: 44)), that were identified in mammalian Neu1 proteins, are indeed, implicated in the regulated nuclear import of Neu1. Importin-α family members are involved in the formation of the NLS receptor complexes that govern the protein transport to the nucleus. Ullman et al., *Cell*, 90:967-970 (1997); and Izaurralde and Adam, *RNA*, 4:351-364 (1998). We have identified importin-α3 as one of the m-Neu interacting proteins by yeast two-hybrid screening.

Neu interactions with Miz1/GBP/PIAS family of proteins

Yeast two hybrid screening revealed that Neu1 interacts with NeuI-1 which is a new splice variant of Miz1/PIASX zinc finger transcription factor. Miz1 is a sequence specific DNA binding protein that functions as a positive-acting transcription factor and interacts directly with homeobox transcription factor Msx2. Wu et al., 1997 *Mech Dev*, 65(1-2):3-17 (1997). Msx1 and Msx2, members of the Msx family of homeobox genes, were found to be important in inductive tissue interactions. Whereas Msx3 was expressed exclusively in the developing nervous system. Wang, et al., *Mech Dev*, 58(1-2):203-15 (1996). Members of the PIAS family, however, regulated DNA binding of STAT transcription factors thereby interfering with the signaling of a variety of

cytokines. Chuang, et al., *Biochem Biophys Res Commun*, 18;235(2):317-20 (1997) and Liu et al., *Proc Natl Acad Sci USA*, 95(18):10626-31 (1998).

Sequence analyses revealed that PIAS1 is identical to Gu/RNA helicase II (Gu/RH-II) binding protein GBP. Valdez et al., *Biochem Biophys Res Commun*, 234(2):335-40 (1997). The GBP regulates proteolytic cleavage of Gu/RH-II which could alter its functions or enzymatic activities or lead to its destruction. These data indicate that the Neu family of proteins could be involved, perhaps through its interactions with various proteins, such as the NeuI-1 protein. Such interactions may be involved in several biologically important regulatory processes including inductive tissue interactions (Miz1), cytokine signaling (PIAS), and RNA processing (GBP).

Neu interactions with ZNF127 zinc finger family of proteins

Yeast two hybrid screening revealed that Neu1 interacts with NeuI-2. This protein is a new member of the ZNF127 zinc finger family of proteins. The ZNF127 gene is localized in Angelman/Prader-Willi region. Disruption of this gene causes a genetic defect related to mental retardation. ZNF127, as well as other genes in this region, were subjected to genomic imprinting (Mowery-Rushton et al., DNA methylation patterns in human tissues of uniparental origin using a zinc-finger gene (ZNF127) from the Angelman/Prader-Willi region *Am J Med Genet*, 61(2):140-6 (1996)). The role of ZNF127 in the development of Angelman/Prader-Willi syndrome, as well as its molecular function, is unknown. The ZNF127 family proteins, however, contain the zinc finger motif, Cx(8)Cx(5)Cx(3)-H, which is characteristic for viral and early immediate genes such as TIS11, ERF-2 (Tabara et al., 1999 pos-1 encodes a cytoplasmic zinc-finger protein essential for germline specification in C. elegans *Development*, 126(1):1-11); and also to RNA binding proteins (Carballo et al. *Science*, 281:1001-1005 (1998)).

Neu interactions with parkin-like proteins

The NeuI-3 protein is another interactor with Neu1 and was found to be similar to the recently described human gene parkin (Kitada, et al., *Nature* 392(6676):605-8 (1998). Mutations in the parkin gene have been shown to result in autosomal recessive

juvenile parkinsonism. The molecular function of the parkin encoded protein is unknown. Phylogenetic analysis reveals that ari and parkin are distant members of a common progeny.

Neu interactions with androgen receptor coregulator ARA54

NeuI-4, another Neu interactor, is identical to ARA54, an androgen receptor coregulator (Kang et al., J. Biol. Chem. 274, 8570-8576; 1999). Furthermore, the RING-zinc finger domain of NeuI-4 has high similarity to *Drosophila* protein ariadne (ari) that could be involved in axonal path-finding. Aguilera, et al., *Genetics* 155(3):1231-44 (1996). Two mammalian homologues of ari have been identified, however, no information is available about molecular mechanisms of the functioning of the ari family of proteins.

Neu function based on the nature of Neu interactors

Based on the nature of Neu1 interactors, it is hypothesized that Neu has the potential to interfere with inductive tissue interactions (NeuI-1/Miz), cytokine signalling (NeuI-1/PIAS), RNA processing (NeuI-1/GBP), early immediate responses (NeuI-2/ZNF127), death of specific cell populations (NeuI-3/parkin), and nuclear hormone receptor signaling and axonal path-finding (NeuI-4/ariadne).

Nucleic Acids

Having identified a number of potential functions for the Neu family of proteins, the described invention seeks to utilize this knowledge to manipulate the various developmental pathways in which Neu functions. As a preliminary step, representative members of the Neu family of proteins have been isolated and purified. Polynucleotide molecules encoding the proteins of the Neu family were then isolated and their sequences are provided below.

Representative polynucleotide molecules encoding members of the Neu family include sequences comprising SEQ. ID. NOs.: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, and 33. Polynucleotide molecules encoding Neu family members

include those sequences resulting in minor genetic polymorphisms, differences between species, and those that contain amino acid substitutions, additions, and/or deletions.

In some instances, one can employ such changes in the sequence of a recombinant Neu to substantially decrease or even increase the biological activity of Neu relative to the wild-type Neu activity. Such changes can also be directed towards endogenous *neu* sequences using, for example, gene therapy methods to alter the gene product. Advantageously, the disclosed sequences can be used to identify and isolate *neu* polynucleotide encoding molecules from suitable vertebrate host cells. Thus, in another embodiment, a method of identifying *neu* polynucleotide molecules is provided.

The nucleotide sequences encoding the neuralized homology repeat domain can be used to identify polynucleotide molecules encoding other proteins of the Neu family. Complementary DNA molecules encoding Neu family members can be obtained by constructing a cDNA library from mRNA of, for example, brain or muscle tissues that are at different developmental stages. DNA molecules encoding Neu family members can be isolated from such a library using the disclosed sequences in standard hybridization techniques or by amplification of sequences using polymerase chain reaction (PCR) amplification.

In a similar manner, genomic DNA encoding Neu can be obtained using probes designed from the sequences disclosed herein. Suitable probes for use in identifying Neu family sequences can be obtained from Neu-specific sequences that are highly conserved regions between mammalian coding sequences. Primers, for example, from the neuralized homology motif domains 1 and 2 are suitable for use in designing PCR primers. Alternatively, oligonucleotides containing specific DNA sequences from a *neu* family coding region can be used to identify related human *neu* genomic and cDNA clones. One of skill in the art will appreciate that upstream regulatory regions of the *neu* family of genes can be obtained using similar methods.

neu family polynucleotide molecules can be isolated using standard hybridization techniques with probes of at least about 7 nucleotides in length and up to and including the full coding sequence. Other members of the *neu* family can be identified using degenerate oligonucleotides capable of hybridization based on the sequences disclosed herein for use PCR amplification or by hybridization at moderate or

greater stringency. The term, "capable of hybridization" as used herein means that the subject nucleic acid molecules (whether DNA or RNA) anneal to an oligonucleotide of 15 or more contiguous nucleotides of SEQ. ID. NOs.: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, and 33.

The choice of hybridization conditions will be evident to one skilled in the art and will generally be guided by the purpose of the hybridization, the type of hybridization (DNA-DNA or DNA-RNA), and the level of desired relatedness between the sequences. Methods for hybridization are well established in the literature. One of ordinary skill in the art realizes that the stability of nucleic acid duplexes will decrease with an increased number and location of mismatched bases; thus, the stringency of hybridization can be used to maximize or minimize the stability of such duplexes. Hybridization stringency can be altered by: adjusting the temperature of hybridization; adjusting the percentage of helix-destabilizing agents, such as formamide, in the hybridization mix; and adjusting the temperature and salt concentration of the wash solutions. In general, the stringency of hybridization is adjusted during the post-hybridization washes by varying the salt concentration and/or the temperature, resulting in progressively higher stringency conditions.

An example of progressively higher stringency conditions is as follows: 2 x SSC/0.1% SDS at about room temperature (hybridization conditions); 0.2 x SSC/0.1% SDS at about room temperature (low stringency conditions); 0.2 x SSC/0.1% SDS at about 42°C (moderate stringency conditions); and 0.1 x SSC at about 68°C (high stringency conditions). Washing can be carried out using only one of these conditions, e.g., high stringency conditions, or each of the conditions can be used, e.g., for 10-15 minutes each, in the order listed above, repeating any or all of the steps listed. However, as mentioned above, optimal conditions will vary, depending on the particular hybridization reaction involved, and can be determined empirically. In general, conditions of high stringency are used for the hybridization of the probe of interest.

Alternatively, polynucleotides having substantially the same nucleotide sequence set forth in SEQ. ID. NOs.: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, and 33 or functional fragments thereof, or nucleotide sequences that are substantially identical to SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27,

29, 31, and 33, can represent members of the Neu family of proteins. By "substantially the same" or "substantially identical" is meant a nucleic acid or polypeptide exhibiting at least 80%, 85%, 90%, 95% or 100% homology to a reference nucleic acid. For nucleotide sequences, the length of comparison sequences will generally be at least 10 to 500 nucleotides in length. More specifically, the length of comparison will be at least 50 nucleotides, at least 60 nucleotides, at least 75 nucleotides, and at least 110 nucleotides in length.

One embodiment of the invention provides isolated and purified polynucleotide molecules encoding Neu proteins, wherein the polynucleotide molecules that are capable of hybridizing under moderate to stringent conditions to an oligonucleotide of 15 or more contiguous nucleotides of SEQ. ID. NOs.: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, and 33, including complementary strands thereto.

DNA sequences of the invention can be obtained by several methods. For example, the DNA can be isolated using hybridization or computer-based techniques which are well known in the art. Such techniques include, but are not limited to: 1) hybridization of genomic or cDNA libraries with probes to detect homologous nucleotide sequences; 2) antibody screening of expression libraries to detect cloned DNA fragments with shared structural features; 3) polymerase chain reaction (PCR) on genomic DNA or cDNA using primers capable of annealing to the DNA sequence of interest; 4) computer searches of sequence databases for similar sequences; and 5) differential screening of a subtracted DNA library.

Screening procedures which rely on nucleic acid hybridization make it possible to isolate any gene sequence from any organism, provided the appropriate probe is available. Oligonucleotide probes, which correspond to a part of the *neu* family of sequences provided herein and encoding a Neu protein family member, can be synthesized chemically. This requires that short, oligo-peptide stretches of the amino acid sequence be known. The DNA sequence encoding the protein can be deduced from the genetic code, however, the degeneracy of the code must be taken into account. It is possible to perform a mixed addition reaction when the sequence is degenerate. This includes a heterogeneous mixture of denatured double-stranded DNA. For such screening, hybridization is preferably performed on either single-stranded DNA or

denatured double-stranded DNA. Hybridization is particularly useful in the detection of cDNA clones derived from sources where an extremely low amount of mRNA sequences relating to the polypeptide of interest are present. In other words, by using stringent hybridization conditions directed to avoid non-specific binding, it is possible, for example, to allow the autoradiographic visualization of a specific cDNA clone by the hybridization of the target DNA to that single probe in the mixture which is its complete complement. (Wallace, *et al., Nucl. Acid Res.*, 9:879, 1981). Alternatively, a subtractive library is useful for elimination of non-specific cDNA clones.

Among the standard procedures for isolating cDNA sequences of interest is the formation of plasmid- or phage-carrying cDNA libraries which are derived from reverse transcription of mRNA which is abundant in donor cells that have a high level of gene expression. When used in combination with polymerase chain reaction technology, even rare expression products can be cloned. In those cases where significant portions of the amino acid sequence of the polypeptide are known, the production of labeled single or double-stranded DNA or RNA probe sequences duplicating a sequence putatively present in the target cDNA can be employed in DNA/DNA hybridization procedures which are carried out on cloned copies of the cDNA which have been denatured into a single-stranded form (Jay, *et al., Nucl. Acid Res.*, 11:2325, 1983).

The nucleotide sequences of the present invention have a myriad of applications. Representative uses of the nucleotide sequences of the invention include the construction of cDNA and oligonucleotide probes useful in Northern, Southern, and dot-blot assays for identifying and quantifying the level of expression of Neu family proteins in a cell. Lack of expression of a Neu protein in tumors, diseased cells or tissues can indicate that measuring the level of Neu expression can provide prognostic markers for assessing the growth rate and invasiveness of a tumor.

In addition, considering the important role of Neu in development and Ca^{2+} signaling, it is thought highly likely that birth defects, degenerative, and psychiatric diseases can result from expression of an abnormal Neu protein. In this case, the Neu protein family can prove highly useful in prenatal screening of mothers and/or for *in utero* testing of fetuses. Also, early diagnosis of degenerative and neurological diseases

can be based on the analyses of changes in Neu expression and mutations in the *neu* genes.

Similarly, the nucleotide sequences can be employed for the construction of recombinant cell lines, ova, and transgenic embryos and animals including dominant-negative and "knock-out" recombinant cell lines in which the regulatory activity of Neu protein is down-regulated or eliminated. Such cells can contain altered Neu coding sequences that result in the expression of a Neu protein that is not capable of enhancing, suppressing or activating transcription of the target gene. The subject cell lines and animals find use in screening for candidate therapeutic agents capable of either substituting for a function performed by Neu or correcting the cellular defect caused by a defective Neu.

The Neu family of proteins presents an attractive set of diagnostic and therapeutic targets, considering the important regulatory role this family of proteins plays in the development function of adult organisms. This important role is reflected by the effects one or defects in a mutant Neu protein can inflict upon an organism. Moreover, with the advances in art of gene therapy progresses, these defects can be correctable *in utero* or in early post-natal life or alternatively through the use of compounds identified in screening assays using Neu proteins. In addition, *neu* polynucleotide molecules can be joined to reporter genes, such as beta-galactosidase, luciferase, or green fluorescent proteins (GFP) and inserted into the genome of a suitable host cell such as an embryonic or tissue specific stem cell by, for example, homologous recombination. Cells expressing *neu* can then be obtained by subjecting the differentiating cells to cell sorting, leading to the purification of a population of *neu* expressing cells. These cells can be useful for studying specific activity of isolated cell populations. Also, these cells can be used to study sensitivity to growth factors or chemotherapeutic agents.

In yet another application of the nucleotide sequence discovery, the technology can be useful in the construction of gene transfer vectors (e.g., retroviral vectors, and the like). In these vectors, the *neu* sequence is often inserted into the coding region of the vector under the control of a promoter. *neu* gene therapy can be used to correct neurological and movement diseases and cancer. For these therapies, gene transfer

vectors can either be injected directly at the site of diseased cells, or the vectors can be used to construct transformed host cells that are then injected at the site of disease.

In one embodiment, a vector comprising a DNA molecule coding a Neu protein is provided. Preferably, a DNA molecule coding a Neu protein is inserted into a suitable expression vector, which is in turn used to transfect or transform a suitable host cell. Exemplary expression vectors for use in carrying out the present invention include a promoter capable of directing the transcription of a polynucleotide molecule of interest in a host cell. Representative expression vectors include both plasmid and/or viral vector sequences. Suitable vectors include retroviral vectors, vaccinia viral vectors, CMV viral vectors, BLUESCRIPT (Stratagene, San Diego, CA) vectors, baculovirus vectors, and the like. In another embodiment, promoters capable of directing the transcription of a cloned gene or cDNA can be inducible or constitutive promoters and include viral and cellular promoters. In particularly preferred embodiments, viral vectors are employed for use in expressing Neu proteins in mammalian cells particularly if *neu* is used for gene therapy.

In some embodiments, it can be preferable to use a selectable marker to identify cells that contain the cloned DNA. Selectable markers are generally introduced into the cells along with the cloned DNA molecules and include genes that confer resistance to drugs, such as neomycin, hygromycin, and methotrexate. Selectable markers can also complement auxotrophies in the host cell. Other selectable markers provide detectable signals, such as beta-galactosidase to identify cells containing the cloned DNA molecules. Advantageously, the selectable markers are amplifiable. Such amplifiable selectable markers can be used to amplify the number of sequences integrated into the host genome.

Antisense

Antisense *neu* nucleotide sequences can be used to block expression of mutant *neu* expression in a variety of cell types. Suitable antisense oligonucleotides are at least 11 nucleotides in length and can include untranslated (upstream or intron) and associated coding sequences. As will be evident to one skilled in the art, the optimal length of an anti sense oligonucleotide depends on the strength of the interaction

between the antisense oligonucleotide and the complementary mRNA, the temperature and ionic environment in which translation takes place, the base sequence of the antisense oligonucleotide, and the presence of secondary and tertiary structure in the mRNA and/or in the antisense oligonucleotide. Suitable target sequences for antisense oligonucleotides include intron-exon junctions (to prevent proper splicing), regions in which DNA/RNA hybrids will prevent transport of mRNA from the nucleus to the cytoplasm, initiation factor binding sites, ribosome binding sites, and sites that interfere with ribosome progression.

Antisense oligonucleotides can be prepared, for example, by the insertion of a DNA molecule containing the target DNA sequence into a suitable expression vector such that the DNA molecule is inserted downstream of a promoter in a reverse orientation as compared to the gene itself. The expression vector can then be transduced, transformed or transfected into a suitable cell resulting in the expression of antisense oligonucleotides. Alternatively, antisense oligonucleotides can be synthesized using standard manual or automated synthesis techniques. Synthesized oligonucleotides are introduced into suitable cells by a variety of means including electroporation, calcium phosphate precipitation, or microinjection. The selection of a suitable antisense oligonucleotide administration method will be evident to one skilled in the art. With respect to synthesized oligonucleotides, the stability of antisense oligonucleotide-mRNA hybrids are advantageously increased by the addition of stabilizing agents to the oligonucleotide. Stabilizing agents include intercalating agents that are covalently attached to either or both ends of the oligonucleotide. In preferred embodiments, the oligonucleotides are made resistant to nucleases by, for example, modifications to the phosphodiester backbone by the introduction of phosphotriesters, phosphonates, phosphorothioates, phosphoroselenoates, phosphoramidates, phosphorodithioates, or morpholino rings.

Protein Production

As would be evident to one skilled in the art, the polynucleotide molecules of the present invention can be expressed in a variety of prokaryotic and eucaryotic organisms. For example, the Neu family of proteins can be expressed in to,

Saccharomyces cerevisiae, filamentous fungi, and bacteria, such as *E. coli* to produce Neu proteins. Similarly, one can express the protein of the described invention in other host cells such as avian, insect, and plant cells using regulatory sequences, vectors, and methods well established in the literature.

Neu proteins produced according to the present invention can be purified using a number of established methods such as affinity chromatography using anti-Neu antibodies coupled to a solid support. Fusion proteins of antigenic tag and Neu can be purified using antibodies to the tag. Optionally, additional purification is achieved using conventional purification means such as liquid chromatography, gradient centrifugation, and gel electrophoresis, among others. Methods of protein purification are known in the art and can be applied to the purification of recombinant Neu described herein.

Amino Acids

In one embodiment, the identification of mammalian Neu genes is provided. Preferably, the mammalian Neu genes have highly conserved sequences across the neuralized homology motif domains at the amino acid level (Neu1, Neu 2, Neu3, and Neu 4). The following Neu polypeptides or proteins have been identified: human neural Neu1 protein of SEQ. ID. NO.: 2, human muscle Neu1 protein SEQ. ID. NO.: 4; human Neu1 alternatively spliced form (h-neu1ΔNHR1) of SEQ. ID. NO.: 6; mouse neural Neu1 protein of SEQ. ID. NO.: 8; mouse muscle Neu1 protein SEQ. ID. NO.: 10; mouse Neu1 alternatively spliced form (m-neu1ΔNHR2A) of SEQ. ID. NO.: 12; mouse Neu1 alternatively spliced form (m-neu1ΔNHR2B) of SEQ. ID. NO.: 14; rat Neu 1 protein SEQ. ID. NO.: 16; rat Neu1 alternatively spliced form (r-neu1ΔNHR2A) of SEQ. ID. NO.: 18; rat Neu1 alternatively spliced form (r-neu1ΔNHR2B) of SEQ. ID. NO.: 20; human Neu2 protein of SEQ. ID. NO.: 22; human Neu2 alternatively spliced form (h-neu2ΔNHR1) of SEQ. ID. NO.: 24; human Neu2 alternatively spliced form (h-neu2ΔNHR2) of SEQ. ID. NO.: 26; rat Neu 2 protein SEQ. ID. NO.: 28; human Neu3 protein of SEQ. ID. NO.: 30; mouse Neu3 protein of SEQ. ID. NO.: 32; and human Neu4 protein (partial) of SEQ. ID. NO.: 34.

The described invention encompasses Neu variants that, for example, are modified in a manner that results in Neu proteins capable of translocating into the nucleus but unable to repress transcription. Fragments of Neu proteins that are capable of transcriptional repression but are incapable of translocating into the nucleus are also encompassed by the present description. Proteins retrieved from naturally occurring materials and closely related, functionally similar proteins retrieved by antisera specific to Neu, and recombinantly expressed proteins encoded by genetic materials (DNA, RNA, cDNA) retrieved on the basis of their similarity to the unique regions in the *neu* family of genes, are also encompassed by the present description.

According to the present description, polynucleotide molecules encoding Neu encompass those molecules that encode Neu proteins or peptides that share identity with the sequences shown in SEQ. ID. NOs.: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, and 34. Such molecules preferably share greater than 30% identity at the amino acid level with the disclosed sequences in Neu. In preferred embodiments, the polynucleotide molecules can share greater identity at the amino acid level across highly conserved regions such as the neuralized homology repeat domains and the RING-zinc finger domains.

It is contemplated that amino acid sequences substantially the same as the sequences set forth in SEQ. ID. NOs.: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, and 34, are encompassed by the described invention. A preferred embodiment includes polypeptides having substantially the same sequence of amino acids as the amino acid sequence set forth in SEQ ID NOs.: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, and 34, or functional fragments thereof, or amino acid sequences that are substantially identical to SEQ ID NOs.: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, and 34. By "substantially the same" or "substantially identical" is meant a polypeptide exhibiting at least 80%, preferably 85%, more preferably 90%, and most preferably 95% homology to a reference amino acid sequence. For polypeptides, the length of comparison sequences will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably 35 amino acids.

Homology is often measured using sequence analysis software (*e.g.*, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Such software matches similar sequences by assigning degrees of homology to various substitutions, deletions, substitutions, and other modifications.

The term "functional fragments" include those fragments of SEQ ID NOs.: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, and 34, or other Neu family members that retain the function or activity of a Neu transcriptional regulator. One of skill in the art can screen for the functionality of a fragment by using the examples provided herein, where full-length Neu transcriptional factors are described. It is also envisioned that fragments of various Neu proteins that inhibit or promote transcription can be identified in a similar manner. Neu transcriptional activity can also be assayed by standard transcription assays.

By "substantially identical" is also meant an amino acid sequence which differs only by conservative amino acid substitutions, for example, substitution of one amino acid for another of the same class (*e.g.*, valine for glycine, arginine for lysine, *etc.*) or by one or more non-conservative substitutions, deletions, or insertions located at positions of the amino acid sequence which do not destroy the function of the protein assayed, (*e.g.*, as described herein). Preferably, such a sequence is at least 85%, more preferably identical at the amino acid level to SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, and 34.

By a "substantially pure polypeptide" is meant a Neu protein that has been separated from components which naturally accompany it. Typically, the polypeptide is substantially pure when it is at least 60%, by weight, free from the proteins and naturally occurring organic molecules with which it is typically associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, Neu protein. A substantially pure Neu polypeptide can be obtained, for example, by extraction from a natural source; by expression of a recombinant nucleic acid encoding a Neu polypeptide; or by chemically synthesizing the protein. Purity can be measured by any appropriate method, *e.g.*, column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis.

A protein is substantially free of naturally associated components when it is separated from those contaminants that accompany it in its natural state. Thus, a protein that is chemically synthesized or produced in a cellular system different from the cell from which it naturally originates will be substantially free from its naturally associated components. Accordingly, substantially pure polypeptides include those derived from eukaryotic organisms but synthesized in *E. coli* or other prokaryotes.

As would be evident to one skilled in the art, the polynucleotide molecules of the present disclosure can be expressed in a variety of prokaryotic and eucaryotic cells using regulatory sequences, vectors, and methods well established in the literature.

Neu proteins produced according to the present invention can be purified using a number of established methods such as affinity chromatography using anti-Neu antibodies coupled to a solid support. Fusion proteins of antigenic tag and Neu can be purified using antibodies to the tag. Optionally, additional purification is achieved using conventional purification means such as liquid chromatography, gradient centrifugation, and gel electrophoresis, among others. Methods of protein purification are known in the art and can be applied to the purification of recombinant Neu described herein.

Construction of interspecies hybrid Neu proteins and hybrid Neu proteins containing one or more domains from another Neu family member are also contemplated. Such hybrid proteins facilitate structure-function analyses. Similarly, hybrid proteins allow for the alteration of Neu activity by increasing or decreasing the transcriptional regulation of target genes. Hybrid proteins of the present invention contain the replacement of one or more contiguous amino acids of the native Neu with the analogous amino acid(s) of Neu from another species or other protein of the Neu family. Such interspecies or interfamily hybrid proteins include hybrids having whole or partial domain replacements. Such hybrid proteins are obtained using recombinant DNA techniques well known by one of skill in the art. Briefly, DNA molecules encoding the hybrid Neu proteins of interest are prepared using generally available methods such as PCR mutagenesis, site-directed mutagenesis, and/or restriction digestion and ligation. The hybrid DNA is then inserted into expression vectors and introduced into suitable host cells.

One embodiment of the present invention involves the isolation of proteins that interact with Neu proteins and regulate Neu protein function or are regulated by Neu. Neu proteins can be used in immunoprecipitation to isolate interacting factors or used for the screening of interactors using different methods of two hybrid screening. Isolated interactors of Neu can be used to modify Neu activity or Neu can be used to modify the activity of interactors. Two hybrid screening has resulted in the isolation of several types of interactors. Sequence analyses showed that all interactors are novel proteins and contain RING-zinc finger domain located in the C-terminus of the protein. NeuI-1 (4 clones) is a novel splice variant (SEQ. ID. NOS.: 35, 36) of zinc finger protein Miz1/PIASXα/ARIP3 (GenBank accession numbers NM_008602; AF077953; AF077954; AF044058). NeuI-2 (3 clones) is a fourth homolog (SEQ. ID. NOS.: 37, 38; GenBank accession number AF277171; AF302084) of zinc finger protein ZNF127 (GenBank accession numbers U19106; U19107). NeuI-3 (9 clones) has highest homology to a human hypothetical protein (GenBank accession number AK001459) and to a *Drosophila* hypothetical protein (AAF56052.2) produced from CG4813 gene of a genomic scaffold (GenBank accession number AE003740) (SEQ. ID. NOS.: 39, 40). NeuI-4 (12 clones) is the homolog of the androgen receptor coactivator ARA54 (SEQ. ID. NO.: 32; GenBank accession number AF060544) (SEQ. ID. NO.: 41, 42).

In still another embodiment, synthetic peptides, recombinantly derived peptides, fusion proteins, chiral proteins (stereochemical isomers, racemates, enantiomers, and D-isomers) and the like are provided which include a portion of Neu or the entire protein. The subject peptides have an amino acid sequence encoded by a nucleic acid which hybridizes under stringent conditions with an oligonucleotide of 15 or more contiguous nucleotides of SEQ. ID. NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33. Representative amino acid sequences of the subject peptides are disclosed in SEQ. ID. NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, and 34. The subject peptides find a variety of uses, including preparation of specific antibodies and preparation of antagonists of Neu activity.

Antibodies

As noted above, the described teachings provide antibodies that bind to Neu. The production of non-human antisera or monoclonal antibodies (e.g., murine, lagomorph, porcine, equine) is well known and can be accomplished by, for example, immunizing an animal with Neu protein or peptides. Additionally, catalytic antibodies to nuclear isoforms of the Neu family of proteins or Neu protein metabolic intermediates that are transported into and out of the nucleus can be generated. For the production of monoclonal antibodies, antibody producing cells are obtained from immunized animals, immortalized and screened, or screened first for the production of the antibody that binds to the Neu protein or peptides and then immortalized. It can be desirable to transfer the antigen binding regions (e.g., F(ab')2 or hypervariable regions) of non-human antibodies into the framework of a human antibody by recombinant DNA techniques to produce a substantially human molecule.

Following synthesis or expression and isolation or purification of a Neu protein or a portion thereof, the isolated or purified protein can be used to generate antibodies and tools for identifying agents that interact with the Neu protein and fragments of the Neu protein. Depending on the context, the term "antibodies" can encompass polyclonal, monoclonal, chimeric, single chain, Fab fragments and fragments produced by a Fab expression library. Antibodies that recognize Neu proteins and fragments of Neu proteins have many uses including, but not limited to, biotechnological applications, therapeutic/prophylactic applications, and diagnostic applications.

For the production of antibodies, various hosts including goats, rabbits, rats, mice, etc. can be immunized by injection with Neu proteins or any portion, fragment or oligopeptide that retains immunogenic properties. Depending on the host species, various adjuvants can be used to increase immunological response. Such adjuvants include, but are not limited to, Freund's, mineral gels such as aluminum hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol. BCG (Bacillus Calmette-Guerin) and Corynebacterium parvum are also potentially useful adjuvants.

Peptides used to induce specific antibodies can have an amino acid sequence consisting of at least three amino acids, and preferably at least 10 to 15 amino acids.

Preferably, short stretches of amino acids encoding fragments of Neu proteins are fused with those of another protein such as keyhole limpet hemocyanin such that an antibody is produced against the chimeric molecule. While antibodies capable of specifically recognizing Neu proteins can be generated by injecting synthetic 3-mer, 10-mer, and 15-mer peptides that correspond to a protein sequence of Neu proteins into mice, a more diverse set of antibodies can be generated by using recombinant Neu proteins, purified Neu proteins, or fragments of Neu proteins.

To generate antibodies to Neu proteins and fragments of Neu proteins, a substantially pure Neu protein or a fragment of Neu protein is isolated from a transfected or transformed cell. The concentration of the polypeptide in the final preparation is adjusted, for example, by concentration on an Amicon filter device, to the level of a few micrograms/ml. Monoclonal or polyclonal antibody to the polypeptide of interest can then be prepared as follows:

Monoclonal antibodies to Neu proteins or a fragment of Neu proteins can be prepared using any technique that provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique originally described by Koehler and Milstein (*Nature* 256:495-497 (1975), the human B-cell hybridoma technique (Kosbor et al. *Immunol Today* 4:72 (1983); Cote et al *Proc Natl Acad Sci* 80:2026-2030 (1983), and the EBV-hybridoma technique Cole et al. Monoclonal Antibodies and Cancer Therapy, Alan R. Liss Inc, New York N.Y., pp 77-96 (1985). In addition, techniques developed for the production of "chimeric antibodies", the splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity can be used. (Morrison et al. *Proc Natl Acad Sci* 81:6851-6855 (1984); Neuberger et al. Nature 312:604-608(1984); Takeda et al. *Nature* 314:452-454(1985). Alternatively, techniques described for the production of single chain antibodies (U.S. Pat. No. 4,946,778) can be adapted to produce Neu protein-specific single chain antibodies. Antibodies can also be produced by inducing *in vivo* production in the lymphocyte population or by screening recombinant immunoglobulin libraries or panels of highly specific binding reagents as disclosed in Orlandi et al., *Proc Natl Acad Sci* 86: 3833-3837 (1989), and Winter G. and Milstein C; *Nature* 349:293-299 (1991).

Antibody fragments that contain specific binding sites for Neu proteins can also be generated. For example, such fragments include, but are not limited to, the $F(ab')_2$ fragments that can be produced by pepsin digestion of the antibody molecule and the Fab fragments that can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries can be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity. (Huse W. D. et al. *Science* 256:1275-1281 (1989)).

By one approach, monoclonal antibodies to Neu proteins or fragments thereof are made as follows. Briefly, a mouse is repetitively inoculated with a few micrograms of the selected protein or peptides derived therefrom over a period of a few weeks. The mouse is then sacrificed, and the antibody producing cells of the spleen isolated. The spleen cells are fused in the presence of polyethylene glycol with mouse myeloma cells, and the excess unfused cells destroyed by growth of the system on selective media comprising aminopterin (HAT media). The successfully fused cells are diluted and aliquots of the dilution placed in wells of a microtiter plate where growth of the culture is continued. Antibody-producing clones are identified by detection of antibody in the supernatant fluid of the wells by immunoassay procedures, such as ELISA, as originally described by Engvall, E., *Meth. Enzymol.* 70:419 (1980), and derivative methods thereof. Selected positive clones can be expanded and their monoclonal antibody product harvested for use. Detailed procedures for monoclonal antibody production are described in Davis, L. et al. Basic Methods in Molecular Biology Elsevier, New York. Section 21-2.

Polyclonal antiserum containing antibodies to heterogenous epitopes of a single protein can be prepared by immunizing suitable animals with the expressed protein or peptides derived therefrom described above, which can be unmodified or modified to enhance immunogenicity. Effective polyclonal antibody production is affected by many factors related both to the antigen and the host species. For example, small molecules tend to be less immunogenic than others and can require the use of carriers and adjuvant. Also, host animals vary in response to site of inoculations and dose, with both inadequate or excessive doses of antigen resulting in low titer antisera. Small doses (ng level) of antigen administered at multiple intradermal sites appears to be most reliable. An effective

immunization protocol for rabbits can be found in Vaitukaitis, J. et al. *J. Clin. Endocrinol. Metab.* 33:988-991 (1971).

Booster injections can be given at regular intervals, and antiserum harvested when antibody titer thereof, as determined semi-quantitatively, for example, by double immunodiffusion in agar against known concentrations of the antigen, begins to fall. See, for example, Ouchterlony, O. et al., Chap. 19 in: Handbook of Experimental Immunology D. Wier (ed) Blackwell (1973). Plateau concentration of antibody is usually in the range of 0.1 to 0.2 mg/ml of serum (about 12μM). Affinity of the antisera for the antigen is determined by preparing competitive binding curves, as described, for example, by Fisher, D., Chap. 42 in: Manual of Clinical Immunology, 2d Ed. (Rose and Friedman, Eds.) Amer. Soc. For Microbiol., Washington, D.C. (1980). Antibody preparations prepared according to either protocol are useful in quantitative immunoassays that determine concentrations of antigen-bearing substances in biological samples; they are also used semi-quantitatively or qualitatively (e.g., in diagnostic embodiments that identify the presence of a Neu protein in biological samples). It is also contemplated that various methods of molecular modeling and rational drug design can be applied to identify additional Neu protein family members, compounds that resemble a Neu protein or fragment or derivative thereof, and molecules that interact with Neu proteins and, thereby modulate their function.

Additional applications

The Neu family of proteins presents an attractive set of diagnostic and therapeutic targets, considering the important regulatory role this family of proteins plays in the development function of adult organisms. This important role is reflected by the effects one or more defects in a mutant Neu protein can inflict upon an organism. Moreover, with the advances in the art of gene therapy, these defects can be corrected *in utero* or in early post-natal life or alternatively through the use of compounds identified in screening assays using Neu proteins.

In some instances, cancer cells, or diseased cells or tissue, can contain a non-functional Neu protein or can contain no Neu protein due to a genetic mutation or

somatic mutations such that these cells fail to stop proliferating and differentiate. For cancers of this type, the cancer cells can be treated in a manner to cause the over-expression of wild-type Neu protein to force differentiation and cease proliferation of the cancer cells. Accordingly, a method of treating cancer is similarly provided.

It is also contemplated that because *neu* family genes control cell proliferation and Ca^{2+} signaling induced transcriptional processes, that manipulating Neu expression and function may be useful in controlling a variety of diseases, a few examples of which include depression, pain, anxiety, neurodegenerative diseases, and cancer.

The practice of the described invention is illustrated in the following non-limiting examples. The examples are provided below are not intended to limit the invention in any way.

EXAMPLE 1

Characterization of *neu1* transcripts

A mouse cDNA library of postnatal day (P) 1 brain (Stratagene, San Diego, CA) was screened with a mouse 0.7 kb EST (GenBank #AA518339) cDNA clone corresponding to a region ranging from about the translation initiation codon to the end of the first neuralized homology repeat domain (NHR1) of h-neu1 (Nakamura et al., 1998).

Sequence analyses, the results of which are shown in Figure 1, revealed that the isolated cDNA clones differed in their 5' regions encoding muscle- and brain-specific m-Neu1 proteins with different N-termini (Fig. 1). In the figure, "A" shows an amino acid sequence comparison of *Drosophila*, human, rat and mouse neu1 proteins. Various domain regions of the proteins are illustrated. The regions *Brain-N*, relating to the neural-specific N-terminal region, and *Muscle-N*, the muscle-specific N-terminal of mammalian neu proteins, are boxed. The *NHR1* and *NHR2* regions, neuralized homology repeat domains 1 and 2; *RZD*, RING zinc finger domain are underlined. *NLS1 and NLS2*, nuclear localization signal sequences, are also shown.

RT-PCR analyses of RNA from mouse and rat brain and skeletal muscle resulted in identification of 6 different Neu1 transcripts in both species (Fig. 1): 1) brain- and muscle-specific transcripts encoding Neu1 with the intact NHR2 domain (574 and 557 amino acids, respectively); 2) brain- and muscle-specific transcripts encoding Neu1 protein isoforms that lack the region between NHR1 and NHR2, the entire NHR2 region and different parts of the linker region preceding the RZD (Neu1-ΔNHR2A, 342 and 325 amino acids and Neu1-ΔNHR2B, 291 and 274 amino acids).

Turning again to Figure 1, the region between asterisks is absent in the splice isoform neu1-ΔNHR2A; the region between the open circles is absent in the splice isoform neu1-ΔNHR2B; the pKB consensus sequence between NHR1 and NHR2; *LRS,* two putative leucine rich sequences in the end of both the NHR; and *SP*, the serine and proline rich repeats between the NHR2 and RZD are boxed. "B" is a schematic representation of the domain structure of different mouse and rat Neu1 isoforms. Structures of the full length Neu1 protein (neu, 574 amino acids) and of two Neu1 isoforms (neu-ΔNHR2A, 342 amino acids, neu-ΔNHR2B, 291 amino acids) are shown. *Lines* indicate to the alternative splicing resulting in the cDNAs encoding neu-ΔNHR2A and neu-ΔNHR2B isoforms. The numbers below the neu-ΔNHR2A and neu-ΔNHR2B correspond to the amino acids of the full length Neu1 protein.

Sequence analyses revealed that alternative splicing in the NHR2 and RZD linker region occurs in-frame and does not affect the intactness of the RING zinc finger structure. Accordingly, both *m-neu1* and *r-neu1* genes encode protein isoforms with one or two NHRs followed by the C3HC4-type RZD in the C-terminus. To date no proteins other than Neu1 proteins have been identified that contain NHR-like domains and the function of the NHR-like domains has not been previously identified. The RING zinc finger motifs are present in many regulatory proteins and have been shown to mediate protein-protein interactions. Saurin et al., *Trends Biochem Sci*, 21:208-214 (1996).

Like h-Neu, m-Neu1 and r-Neu do not have sequences that are similar to the Lys-rich nuclear localization signal (NLS) in d-Neu. However, two smaller clusters of Arg and Lys rich amino acids (HKAVKAR (SEQ ID NO: 43) at 80-85 and RLKITKK (SEQ ID NO: 44) at 107-113) are present in the NHR1 of mouse, rat, and human Neu1

that resemble the phosphorylation consensus sequence of NLS, m-Neu1, r-Neu, and h-Neu proteins, which are rich in Ser and Thr residues. The presence of these regions suggests that Neu1 is regulated by phosphorylation. The region between NHR1 and NHR2 contains also a putative protein kinase B/AKT phosphorylation site RPRSFT (SEQ ID NO: 45) which is similar to the respective consensus sequence RXRXXS/T (SEQ ID NO: 46). Datta, et al., *Genes and Dev*, 13:2905-27 (1999).

The region between NHR2 and RZD of m-Neu1, r-Neu, and h-Neu contains two imperfect repeats of Pro, Ser, and Thr residues with the consensus sequence S/TXPXSPXSXPXSPXXXGXXX(X)SD (SEQ ID NO: 47) where X denotes any amino acid. It is interesting to note that this SP repeat of mammalian Neu1 proteins is not similar to any known protein motifs.

EXAMPLE 2

Developmentally regulated expression of *neu1* mRNA in the mouse and rat skeletal muscle and brain

An RNase protection analyses (RPA) was used to determine the levels of *m-neu1* and *r-neu1* mRNAs in the developing and adult brain and non-neural tissues (Fig. 2).

Total RNA was isolated from the mouse and rat brain regions, which are indicated (A) and (B) respectively in Figure 2, and non-neural tissues. Levels of *neu1* transcripts were analyzed by RNase protection assays. The cRNA probes used for detection of *m-neu1* transcripts were complementary to a region encoding the muscle-specific (Figure 2, *left* panel) or the brain-specific (Figure 2, *right* panel) N-terminus and first half NHR1 of *m-neu1*. For *r-neu1*, the cRNA probe was complementary to a region encoding the second half of NHR2 up to the stop codon. Specific protected fragments are indicated on the right of each panel.

Bottom panels of Figure 2 show the levels of GAPDH mRNA in the RNA samples. The nomenclature used to identify each sample is as follows: *neu-M*, indicates a muscle-specific *m-neu1* transcript; *neuB*, indicates a brain-specific *m-neu1* transcript; *neu-B/M* indicates the total pool of muscle- and brain-specific *r-neu1* transcripts. Further, *E*, denotes "Embryonic day"; *P*, denotes "postnatal day"; *ad*, denotes "adult"; *th*, denotes "thymus"; *he*, denotes "heart"; *lu*, denotes "lung"; *sp*, denotes "spleen"; *ki*,

denotes "kidney"; *li*, denotes "liver"; *mu*, denotes "skeletal muscle"; *gu*, denotes "gut"; *plac*, denotes "placenta"; *ctx*, denotes "cerebral cortex"; *cbl*, denotes "cerebellum"; *hc*, denotes "hippocampus"; *str*, denotes "striatum"; *mid*, denotes "ventral midbrain"; *hth*, denotes "hypothalamus"; *col*, denotes "colliculi"; *thal*, denotes "thalamus"; *pons*, denotes "pons"; *olf*, denotes "olfactory bulb"; *med*, denotes "medulla"; *pit*, denotes "pituitary"; and *tRNA*, denotes "yeast tRNA", which was used as a negative control.

The overall highest expression levels of *neu1* mRNA were seen in the adult skeletal muscle and brain. In the skeletal muscle, *neu1* mRNA levels were undetectable during embryonic development, low at birth and upregulated during postnatal development reaching the highest levels in the adult. Other non-neural tissues (heart, kidney, liver, lung, thymus, and spleen) except for the adult heart and testis did not express *neu1* transcripts or the levels were below the detection limit of the RPA. In the brain, low levels of *neu1* mRNA were detected at embryonic day (E) 13 and the expression increased progressively reaching the highest levels in the adult. *neu1* expression levels were high in the cerebral cortex, hippocampus, and striatum and substantially lower in the olfactory system, thalamus/hypothalamus, midbrain, cerebellum, pons, and medulla.

neu1 expression levels were specifically analyzed during postnatal development of cerebral cortex and cerebellum. In both of these brain regions, low levels of *neu1* mRNA were observed in postnatal day (P) 1 whereas by two weeks after birth the expression levels increased significantly reaching the peak levels in the adult animal. High levels of *neu1* mRNA expression were also detected in the adult dorsal root ganglia and moderate levels in the adult spinal cord. In all the tissues predominant *neu1* transcripts contained intact NHR2 domain, whereas the levels the *neu1* mRNAs lacking the NHR2 (*neu1-ΔNHR1A* and *neu1-ΔNHR1B*) were below 5% of all *m-neu1* transcripts. Presented data show that *neu1* is highly expressed during mouse and rat postnatal development and that the expression is confined to skeletal muscle and the nervous system.

EXAMPLE 3

Neuronal expression and dendritic localization of mammalian *neuralized* mRNA

In situ hybridization analysis was used to study the cellular localization of *neu1* mRNA expression in the embryonic and adult mouse brain. The results are presented in Figure 3.

Shown are dark-field emulsion autoradiographs obtained after hybridization of coronal sections of mouse brain with the [^{35}S]-labeled *m-neu1* cRNA probe corresponding to the first neuralized homology region (NHR1) of neu1. *m-neu1* mRNA-specific labeling is shown at embryonic day 17 (*E17*, upper panel), at postnatal day 7 (*P7*, middle panel) and adult (*AD*, lower panel) in different brain structures. Exposure time was 3 weeks for adult and P7 sections and 6 weeks for E17 sections. As shown in the figure, *BG*, denotes "basal ganglia"; *Cx*, denotes "cerebral cortex"; *Th*, denotes "thalamus"; *PTec*, denotes "pretectum"; *SC*, denotes "superior colliculi"; *IC*, denotes "inferior colliculi"; *Cb*, denotes "cerebellum"; *Me*, denotes "medulla"; *Pit*, denotes "pituitary"; *P*, denotes "pons"; *Hc*, denotes "hippocampus"; *Am*, denotes "amygdala"; and *Pn*, denotes "Pontine nuclei".

Results of these analyses supported the conclusions drawn from the RPA studies discussed in Example 2. *Neuralized1* mRNA-specific labeling was not detected at E13 and E15 brain, because of very low expression levels. This result was supported by the related data produced from the RPA. At E17, all brain regions, except for the cerebral cortex, hippocampus, and cerebellum, were found to express low levels of *m-neu1* mRNA. In the brain, *m-neu1* expression was confined to the regions containing postmitotic neurons and was not present in ventricular and subventricular zones that contain proliferating neural stem and progenitor cells.

At P7, the levels of *neu1* mRNA increased significantly in the basal ganglia, amygdala, hypothalamus, and hippocampus. Low levels were present in the cerebral cortex and brainstem. In the adult, widespread expression of *m-neu1* mRNA was observed throughout the brain particularly in the cerebral cortex, hippocampal formation, the basal ganglia, amygdaloid, hypothalamus, and pontine nuclei and in the cerebellum, while lower levels were seen in the mesencephalon and medulla oblangata. *m-neu1* mRNA-specific signal was not detected in most of the thalamic nuclei.

A more detailed analysis of *neu1* expression was carried out in rat brain and the data is shown in Table 2 and in Figures 4, 5, and 6.

Figure 4 shows the results of an *in situ* hybridization analysis of *neu1* mRNA expression in adult rat brain. The coronal sections (*A-H*) correspond to the levels from Bregma in the atlas of Paxinos and Watson (Paxinus and Watson, 1986) as indicated by numbers in the left part of the bottom of each autoradiograph. *CTX*, denotes "cerebral cortex"; *CPu*, denotes "caudate putamen"; *Pir*, denotes "piriform cortex"; *LS*, denotes "lateral septum"; *HDB*, denotes "nucleus of the horizontal limb of the diagonal band of Broca"; *VDB*, denotes "nucleus of the vertical limb of the diagonal band of Broca"; *GP*, denotes "globus pallidus", *BSTM* and *BSTL*, denotes "bed nucleus of the stria terminalis, medial and lateral division respectively"; *MHb*, denotes "medial habenular nucleus"; *Rt*, denotes "reticular thalamic nucleus"; *AH*, denotes "anterior hypothalamic area"; *Zi*, denotes "zona incerta"; *Am*, denotes "amygdaloid nuclei"; *VMH*, denotes "ventromedial hypothalamic nucleus"; *DMD*, denotes "dorsomedial hypothalamic nucleus"; *DG*, denotes "dentate gyrus"; *CA1* and *CA3*, denotes "pyramidal layers of the hippocampus"; *Ent*, denotes "entorhinal cortex"; *PAG*, denotes "periaqueductal gray"; *Sc*, denotes "superior colliculus"; *S*, denotes "subiculum"; *Pn*, denotes "pontine nuclei"; *GL*, denotes "granular layer of cerebellum"; *PL*, denotes "Purkinje cell layer of cerebellum"; *ECu*, denotes "external cuneate nucleus"; *Ve*, denotes "vestibular nuclei"; *Gi*, denotes "gigantocellular reticular nucleus"; *Sp5*, denotes "spinal trigeminal nucleus": *PCRt* and *IRt*, denote "parvicellular reticular nuclei" and "intermedial reticular nuclei", respectively; *LPGi*, denotes "lateral paragigantocellular nucleus"; *LRt*, denotes "lateral reticular nucleus"; and *Sol*, denotes "solitary nucleus". The scale bar corresponds to 5mm in length.

Figure 5 shows expression of *neu1* mRNA in an adult rat nervous system. Dark-field *in situ* hybridization autoradiographs showing labelling in *A*, the cerebral cortex; *B*, *CA1-CA3*, hippocampal subfields, and in the dentate gyrus *(DG)* including the strata molecular *(Mol)*, lacunosum molecular *(Lmol)* and radiatum *(Rad)*; *C*, substantia nigra compact part *(SNc)* and reticular part *(SNr)*; *D*, medial habenular nucleus *(MHb)*; *E*, locus coeruleus *(LC)*; *F*, in the molecular *(Mol)* and granular *(Gr)* layer of cerebellar cortex; *G*, in the layers of the retina: ganglion cell layer *(GCL)*, inner plexiform layer

(IPL), inner nuclear layer *(INL),* outer plexiform layer *(OPL),* outer nuclear layer *(ONL),* pigmental epitelial *(PE); H,* dorsal root ganglia *(DRG).* Roman numbers in *A* indicate the various layers of cerebral cortex; *CC,* corpus callosum; *Hil,* hilus; *WM,* white matter. The scale bars correspond lengths of 200 μm in A and 400 μm in B-H.

Figure 6 shows cellular localization of *neu1* mRNA in an adult rat nervous system. Bright-field *in situ* hybridization microphotographs showing labelled cells in *A,* in the layer II of the cerebral cortex at the level of the somatosensory cortex *(CTX); B,* dentate gyrus *(DG)* of hippocampus; *C, CA3* layer of the hippocampus; *D,* polymorph layer of dentate gyrus *(PoDG); E,* lateral septum *(LS); F,* substantia nigra compact part *(SNc); G,* globus pallidus *(GP); H* and *I,* spinal cord at L3 level, respectively in the layers 9 and 2-3 *(Sc); J,* dorsal root ganglia *(DRG); K,* glia limitans *(GL); L,* pineal gland *(Pi).* Note in *G,* the cells in the globus pallidus *(GP)* do not express *neu1* mRNA. *Mol,* stratum moleculare; *Hil,* hilus of the hippocampus; *Rad,* stratum radiatum; *Or,* stratum oriens. The scale bar corresponds to 25 μm in length.

Overall, although the *neu1*-specific labeling was distributed broadly throughout the brain, more predominant expression was confined to forebrain structures and the levels were lower in caudal regions of the brain, with the exception of some nuclei (Table 2). In the olfactory system the anterior olfactory nucleus showed *neu1*-specific signal. In the cerebral cortex *neu1* mRNA expression levels were particularly high (Fig. 4A-F, 5A, 6A). In the cerebral cortex, highest levels of *neu1* mRNA expression were found in layer I-III, whereas expression gradually decreases from layer IV to layer VI. Dense labeling was seen in layers I–III, and in scattered neurons of layer V, moderate levels in layers V and VI, and low levels in layer IV. (Fig. 5A, 6A) Interestingly, labeling in layer I did not cover diffuse cell bodies, suggesting dendritic localization of *neu1* mRNA. Piriform and entorhinal corticies were also labeled with *neu1*-specific signal.

In the hippocampus, neurons of the granular layer of dentate gyrus and the pyramidal layers CA1-CA3 showed moderate-high labeling (Fig. 4C-E, 5B, 6B-D). Neurons of the hilus and subiculum expressed low levels of *neu1* mRNA. The most interesting observation regarding *neu1* mRNA localization in the adult brain was the clear dendritic localization of the transcripts in the hippocampus. The *neu1*-specific

labeling over the molecular layer of the dentate granule cells was uniformly distributed (Fig. 4C-E). Examination of emulsion-dipped sections showed that this labeling did not cover cell bodies and extended up to the hippocampal fissure, showing that *neu1* mRNA is localized throughout the entire dendritic tree of dentate granule cell layer (Fig. 5B, 6B). The strata oriens and radiatum of CA1-CA3 of hippocampus, corresponding to the dendrites of these regions, were also labeled by *neu1* cRNA but the levels were significantly lower than in the molecular layer of the dentate gyrus (Fig. 4C-E, 5B, 6C).

In the basal ganglia, *r-neu*1 cRNA was detected in caudate putamen and accumbens but not in the globus pallidus (Fig. 4A, B, 6G). Moderate to high levels of *neu1* mRNA were seen in all the amygdaloid nuclei (Fig. 4C-D), the interstitial nucleus of the posterior limb of anterior commissure and the bed of stria terminalis of extended amygdala (Fig. 4B). In the septum, high levels of labeling were seen in the cells of lateral septum (Fig. 4A; 6E), and very low levels in the vertical and horizontal limb of the diagonal band of Broca. In the hypothalamus, the supraoptic, suprachiasmatic, supramammilary, and ventromedial nuclei expressed moderate levels, and several other nuclei expressed low levels of *neu1* mRNA (Fig. 4B-E). In the thalamus, the medial habenula expressed high levels of *neu1* mRNA, while moderate levels were observed in the reticular thalamic nucleus and low levels in a few other nuclei of the thalamus, such as the zona incerta, lateral habenula, mediodorsal thalamic nuclei, laterodorsal thalamic nuclei, paraventricular thalamic nucleus, rheuniens, rhomboid thalamic nuclei, and medial pretectal nucleus (Table 1, Fig. 4C-D, 5D). In the mesencephalon, the neurons of the substantia nigra, colliculi superior, ventral tegmental area, periaqueductal gray, interpeduncular nucleus, oculomotor nucleus, and Raphe nucleus showed low levels of *neu1*-specific signal (Fig. 4F, 5C, 6F). The granule cell layer of the cerebellum was strongly labeled, but the Purkinje cells and deep cerebellar nuclei were not (Fig. 4G, H, 5F). Within myelenchepalon, the locus coeruleus, and the pontine, trapezoid body, facialis, pontine reticular, and rostroventrolateral reticular nuclei were labeled with moderate density with *r-neu1* cRNA (Table 1, Fig. 4F, 5E). Beyond this, there was low-density labeling in the trigeminal, facial, solitary, and hypoglossal nuclei (Fig. 4G, H). In addition, *neu1* cRNA labeling was evident at low levels or in association with

fewer cells in several other nuclei, such as the vestibular, cochlear, paragigantocellular, and abducens nuclei (Table 1).

In the retina, labeling of *neu1* mRNA was strong in the inner nuclear layer, with the highest intensity in the outer border. Diffuse labeling was also seen in the ganglion cell layer and both in the inner and outer plexiform layers (Fig. 5G). Cells in the pineal gland expressed high levels of *neu1* mRNA (Fig. 6L). Finally, labeling was relatively low in the cells of the spinal cord (Fig. 6H, I), in contrast to the neurons of dorsal root ganglia, which expressed high levels of *neu1* mRNA (Fig. 5H, 6J).

At the cellular level, *m-neu1* mRNA expression was predominant in cells displaying neuronal profile (large cells with weakly stained nuclei) and not in the cells with glial profile (small cells with strongly stained nuclei). In addition, *neu1* mRNA expression was not detected in the white matter, where neuronal cells are absent. One of the few exceptions of non-neuronal *neu1* mRNA expression was glia limitans that displayed dense labeling (Fig. 6K).

Using *in situ* hybridization in combination with immunocytochemistry for NeuN, a Neuron-specific protein, we found that clusters of silver grains, reflecting the presence of *neu1* mRNA, were located over the perikarya of NeuN-positive cells in all the brain regions examined, such as cerebral cortex, hippocampal formation, striatum, hypothalamus nuclei, amygdaloid complex, and cerebellum. Figure 7 shows the results of neuronal expression studies of *neu1* mRNA levels in adult rat brain. In the figure, *neu1* mRNA was visualized as autoradiographic grains whereas NeuN was shown as peroxidase staining resulting in a yellow color. The two labelings co-localized with all the cells. As shown in the figure: *A,* layer II of cerebral cortex *(CTX); B, CA1* layer of the hippocampus; *C,* polymorph layer of the dentate gyrus *(PoDG)* and *CA3* layer of the hippocampus; *D,* caudate putamen *(CPu); E,* reticular thalamic nucleus *(Rt);* and *F,* ventromedial hypothalamic nucleus *(VMH)*, were studied. Note that neurons (NeuN-positive cells) of the ventral posterolateral thalamic nucleus *(VPL)* in *E* do not express *neu1* mRNA .

By evaluation the proportion of cells positive for both *neu1* mRNA and NeuN out of the total number of NeuN-containing neurons, it was found that all the neurons in the layers II and III of the cerebral cortex expressed *neu1* mRNA. In the layer IV eighty

percent and layers V-VI about ninety percent of neurons expressed *neu1* mRNA. In several other brain regions examined, such as hippocampal formation, striatum, reticular thalamic nucleus, hypothalamic nuclei, showing that the majority of the neuronal cells express *neu1* mRNA. In contrast, neurons in the globus pallidus and in several thalamic nuclei, such as ventral posterolateral and posteromedial nuclei, did not express *neu1* mRNA (Fig. 7).

Neuronal activity has been shown to regulate the dendritic localization of the immediate-early gene *arc* mRNA. Steward et al., *Neuron*, 21:741-51 (1998); Guzovsky et al., *Nat Neurosci*, 2:1120-1124 (1999). To examine if *neu1* mRNA dendritic localization is regulated by neuronal activity, the effect of kainate-induced seizures on the expression and localization of *neu1* mRNA was studied (Fig. 8). At 24 hours after the injection of kainate, *neu1* mRNA levels decreased in all the regions of the hippocampus. The levels of *neu1* mRNA are significantly downregulated in the granular and molecular layers of dentate gyrus of adult rat brain at 24 hours after the treatment with kainic acid (B and B1), as compared to control brains (A and A1). Regions analyzed are denoted as follows: *CTX*, cerebral cortex; *DG*, dentate gyrus; *Am*, amygdala; *Pir*, piriform cortex; *VMH*, ventromedial hypothalamic nucleus; and *Mol*, for the molecular layer of the hippocampus. Particularly pronounced was the reduction of labeling in the molecular layer of the dentate gyrus. Similarly, but less consistently, a down-regulation of *neu1* mRNA was observed in the cerebral cortex.

EXAMPLE 4

Neu1 protein isoforms exhibit transcriptional repressor activities

The ability of m-Neu1 to act as a transcriptional regulator was studied using a chloramphenicol acetyl-transferase (CAT) assay. Several transcriptional regulators, such as the polycomb group-related transcriptional regulator MEL18, the MDM2 proto-oncogene, breast and ovarian cancer susceptibility gene BRCA1 and MAT1, a subunit of TFIIH basal complex factor contain RING finger motifs. Kanno et al., *EMBO J*, 14:5672-5678 (1995); Leveillard and Wasylyk, *J Biol Chem*, 272:30651-30661 (1997); Pao et al., *Proc Natl Acad Sci USA*, 97:1020-1025 (2000); and Fesquet et al., *Oncogene*, 15:1303-1307 (1997). Furthermore, the earlier studies proposed d-Neu to function as a

DNA-binding transcription factor. Boulianne et al., *EMBO J*, 10:2975-2983 (1991) and Price et al., *EMBO J*, 12:2411-2418 (1993).

The effect of m-Neu1 on transcriptional activity was studied using a chloramphenicol acetyl-transferase (CAT) assay and the following promoters: 1) TATA-box containing: 1.0 kb BDNF-I-CAT, 0.3 kb BDNF-II-CAT, 0,7 kb BDNF-IV CAT, 0.4 kb NF-L, 1.0 kb GAP-43, 2) TATA-less promoters with putative initiators (Inr): 0.4 kb BDNF-III-CAT, 0.4 kb LNGFR-CAT, and 0.3 kb ME1-CAT. Timmusk et al., *Neuron*, 10:475-489 (1993); Reeben et al., *J Neurosci Res*, 40:177-188 (1995); Chiaramello et al., *J Biol Chem*, 271:22035-22043 (1996); Metsis et al., *Gene*, 121:247-254 (1992); and Shain et al., *Nucleic Acids Res*, 23:1696-1703 (1995). Data from the study is shown in Figure 9.

Figure 9 show that Neu1 represses transcription from various promoters in transient expression assays. The data for this figure was gathered from Neuro2A cells that were co-transfected with 0.5 μg of various reporter plasmids containing different promoters driving the epression of CAT and pRcCMV expression plasmid (1.0 μg) without any cDNA sequence (control) or containing *m-neu1* cDNAs encoding the full length protein (*neu-FL*) or the m-Neu-1-ΔNHR2A *(neuΔNHR2A)* isoform lacking the region between NHR1 and RZD, using FuGENE-6 transfection system. The pON260 expression plasmid (0.1μg) encoding β-galactosidase was included in the transfections to normalize the transfection efficiencies. The CAT activity is defined as 100 for each reporter when cotransfected with the pRcCMV parental expression plasmid. When cotransfected with expression plasmids containing *m-neu1* cDNAs, the CAT activities are expressed relative to the value obtained by cotransfection of each reporter plasmid and the parental pRcCMV expression plasmid. The data shown are representative of at least three independent experiments. Error bars represent the S.E. *BDNF-I*, 1.0 kb BDNF promoter I, *BDNF II*, 0.3 kb BDNF promoter II; *BDNF III*, 0.4 kb BDNF promoter III; *BDNF IV*, 0.7 kb BDNF promoter IV; *NF-L*, 0.4 kb NF-L promoter; *GAP-43*, 1.0 kb GAP-43-promoter, *LNGFR*, 0.4 kb LNGFR promoter; *ME1*, 0.3 kb ME1 promoter.

The full-length and the isoform of m-Neu1-ΔNHR2A significantly reduced the activity of all of these promoters in Neuro-2A cells (Fig 9). CAT assays performed in

other cell types (mouse teratocarcinoma PCC7, rat astrocytoma C6, human breast ductal carcinoma BT-549, human primary osteogenic sarcoma Saos2, human cervix carcinoma C-33A) or using a different reporter (RARβ promoter driven lac Z) gave similar results (data not shown), suggesting that the transcriptional repressor activity of m-Neu1 does not depend on the reporter and cellular context.

Following the identification of m-Neu1's activity as a transcriptional regulator, identification of the repression domains of m-Neu1 was performed. Expression plasmids were generated encoding full-length m-Neu1 or individual domains of m-Neu1 fused to Gal4 DNA-binding domain and tested their activities by CAT assays. The data is presented in Figure 10.

Figure 10 shows that neuralized homology repeat domains of Neu1 mediate the transcriptional repression when fused to the DNA binding domain of Gal4. Neuro2A cells were cotransfected with Gal4TK-CAT reporter plasmid containing five Gal4 binding sites in front of TK promoter driving the expression of CAT (0.5 μg) and pBIND expression plasmid (1.0 μg) without any cDNA sequence (G4) or containing different regions of m-neu1 cDNA fused in-frame to Gal4 DNA-binding domain using FuGENE-6 transfection system. The designations of the constructs are shown on the *left* of Figure 10. The CAT activities are expressed relative to the value obtained by cotransfection of the reporter plasmid and the parental pRcCMV expression plasmid which was set 100. The data shown are representative of at least three independent experiments. Error bars represent the S.E.

The co-transfection of full-length m-Neu1 fused to Gal4 (Gal4-m-Neu1) with the Gal4-TK-CAT resulted in a concentration-dependent repression of the CAT activity (Fig. 10). m-Neu1 isoforms that lack the NHR2 and the region preceding the RZD also displayed transcriptional repressor activities when fused to Gal4 DBD (Fig. 10). Extensive deletion analyses showed that NHR1 and NHR2 domains possess transcriptional repressor activity, whereas neural and muscle-specific N-termini (amino acids 1-60), RZD (amino acids 509-574) and the serine-proline rich linker region joining RZD to NHR2 (amino acids 439-510) did not affect transcription (Fig. 10). Interestingly, individual NHR1 and NHR2 displayed even stronger repressor activities than the full-length m-Neu1. These data show that m-Neu1 acts as a transcriptional

repressor when tethered to a promoter via a heterologous DNA binding domain (DBD). To summarize, m-Neu1 represses the activity of both TATA and TATA-less promoters in transient expression assays.

EXAMPLE 5
Nucleocytoplasmic shuttling of Neu1 protein

The function of Neu1 as a transcriptional repressor implies its nuclear localization. A putative lysine rich nuclear localization signal (NLS) that is present in d-Neu protein (d-NLS) is not conserved in mammals. Boulianne et al., *EMBO J*, 10:2975-2983 (1991) and Price et al., *EMBO J*, 12:2411-2418 (1993). To study the subcellular localization of m-Neu1 protein expression constructs were generated encoding tagged m-Neu-fusion proteins. The expression of these vectors was analyzed in Neuro2A cells.

The results from this work is shown in Figure 11. This figure shows the subcellular localization of m-Neu1-FLAG and m-Neu1-EGFP fusion proteins in Neuro2A cells. Neuro2A cells were transfected expression plasmids encoding m-Neu1-FLAG (A, E, F) and different m-Neu1-EGFP (B, C, D) fusion proteins or with parental pFLAG (G) and pEGFP-C3 (H) expression plasmids. Numbers indicate the amino acids of Neu1 fused to the C-terminal of FLAG and EGFP. Presented are images of FLAG immunofluorescence using anti-FLAG antibody *(A, E, F, G)* and direct fluorescence of EGFP fusion proteins *(B, C, D, H)*.

More specifically, *A,* shows the Neu1-FLAG fusion protein is localized either in the cytoplasm or in the nucleus; *B,* shows that Neu1/120-EGFP fusion protein containing amino acids 1-120 of Neu1 exhibits constitutive nuclear localization in all cells; *C,* shows that the Neu1-60/120-EGFP fusion protein is seen exclusively in the nucleus revealing that the first 60 amino acids of NHR1 are sufficient for nuclear localization of Neu1; in *D,* the Neu1-1/60-EGFP fusion protein containing the N-terminal 60 amino acids of the brain- specific isoform of m-Neu1 shows localization that is identical to *H,* localization of EGFP synthesized from the parental pEGFP-C3 expression plasmid; *E,* inhibition of nuclear export of Neu1-FLAG fusion protein by treatment of Neuro2A cells with leptomycin B (10ng/ml) for 12 hours or *F,* 2 days of RA and dBcAMP mediated differentiation leads to predominant nuclear localization of

Neu1-FLAG fusion protein; *G,* FLAG synthesized from parental plasmid is distributed evenly in the cell.

The fusion protein m-Neu1-FLAG comprised of the full-length m-Neu1 with N-terminal FLAG tag showed either predominantly nuclear (~40% of the positive cells) or predominantly cytoplasmic (~60% of the positive cells) distribution. If localized in the cytoplasm, m-Neu1-FLAG fusion protein was observed in the form of granular speckles in the perinuclear area, in the vicinity of plasma membrane and in neurites (Fig. 11A).

m-Neu1 does not have sequences that are similar to the lysine-rich d-NLS, however, two smaller clusters of arginine and lysine rich amino acids (HKAVKAR (SEQ ID NO: 43) at 80-85 and RLKITKK (SEQ ID NO: 44) at 107-113) are present in the NHR1 of m-Neu1 (Fig. 1). To investigate whether these putative NLSs are functional, the subcellular localization of m-Neu1 deletion mutants fused to the C-terminus of EGFP were analyzed in living cells. Neu-1/120-EGFP fusion protein containing the first 120 amino acids of m-Neu1 displayed nuclear localization in virtually all the cells (100%) (Fig. 11B). Deletion of the first 60 amino acids from the N-terminal region of m-Neu1 up to the NHR1 domain (Neu-60/120-EGFP) did not change the predominant nuclear localization of the Neu-1/120-EGFP fusion protein (Fig. 11C). The N-terminal region of Neu1 fused to EGFP (Neu1/120-EGFP) displayed overall cellular distribution of the fusion protein (Fig. 11D) which was identical to the localization of EGFP (Fig. 11H). These results showed that the region containing the first 60 amino acids of NHR1 (amino acids 60-120) including two putative NLS domains are sufficient for nuclear import of m-Neu1.

Localization of m-Neu1 fusion proteins in the nucleus and cytoplasm suggest that m-Neu1 transport could be the subject of regulation. Various proteins have been shown to be exported from the nucleus by the CRM1/exportin1-related export pathway that is blocked by the antibiotic leptomycin B (LMB), a specific inhibitor of nuclear export mediated by leucine-rich nuclear export signals (NES). Hood and Silver, *Curr Opin Cell Biol*, 11:241-247 (1999); Izaurralde and Adam, *RNA*, 4(:351-364 (1998); Ullman et al., *Cell*, 90:967-970 (1997); Weiss (1998); Fornerod et al., *Cell*, 90:1051-1060 (1997); Fukuda et al., *Nature*, 390:308-311 (1997); Kudo et al., *Exp Cell Res*, 242:540-547 (1998); Kudo et al., *Proc Natl Acad Sci USA*, 96:9112-9117 (1999); and

Nishi et al., *J Biol Chem*, 269:6320-6324 (1994). m-Neu1 protein contains two putative leucine-rich sequences, one in the end of each of the NHR (Fig. 1), that are similar to the identified NES in different proteins. Kogerman et al., *Nat Cell Bio*, 1:312-319 (1999); Taagepera et al., *Proc Natl Acad Sci USA*, 95:7457-7462 (1998); Ullman et al., *Cell*, 90:967-970 (1997); and Yamaga et al., *J Biol Chem*, 274:28537-28541 (1999). In view of these results, the effect of LMB on the localization of m-Neu-FLAG in Neuro2A cells was next studied.

Treatment of Neuro2A cells transfected with m-Neu1-FLAG expression construct with LMB (10ng/ml) resulted in exclusively nuclear localization of m-Neu1-FLAG at 12 hours post-treatment in virtually all the cells (Fig. 11E). These results suggest that m-Neu1 protein shuttles between nucleus and cytoplasm, and that the CRM1/exportin1-related pathway is involved in nuclear export.

Interestingly, the number of cells with nuclear localization of m-Neu1-tagged protein increased substantially (from 45% to 80%) 12 hs after the RA- and cAMP-mediated neuronal differentiation (Fig. 11F). It suggests that neuronal differentiation changes the mechanisms that are responsible for the translocation of m-Neu1 protein in Neuro2A cells.

EXAMPLE 6

Characterization of Neu1 homologs Neu2, Neu3 and Neu4

Screening of cDNA libraries and RT-PCR amplification has resulted in isolation of several homologs of mammalian *neu1* genes. Polynucleotide sequences of the following homologs are presented as SEQ. ID. NOS.: human *neu2* cDNA of SEQ. ID. NO.:21; human *neu2* alternatively spliced form *h-neu2-ΔNHR1* of SEQ. ID. NO.:23; human *neu2* alternatively spliced form *h-neu2-ΔNHR2* of SEQ. ID. NO.:25; rat *neu2* cDNA SEQ. ID. NO.: 27; human *neu3* cDNA of SEQ. ID. NO.: 29; mouse *neu3* cDNA of SEQ. ID. NO.: 31; and human *neu4* cDNA (partial) of SEQ. ID. NO.: 33. Corresponding peptide sequences are presented as SEQ. ID. NOs: human Neu2 protein of SEQ. ID. NO.: 22; human Neu2 alternatively spliced form h-neu2-ΔNHR1 of SEQ. ID. NO.:24; ; human Neu2 alternatively spliced form h-neu2-ΔNHR2 of SEQ. ID. NO.:26; rat Neu2 protein SEQ. ID. NO.: 28; human Neu3 protein of SEQ. ID. NO.: 30;

mouse Neu3 protein of SEQ. ID. NO.: 32; and human Neu4 protein (partial) of SEQ. ID. NO.: 34. All the mammalian Neu proteins show significant homology in the NHR and Ring zinc finger domains.

Alignment of neuralized homology motifs of human Neu1, Neu2, and Neu3 proteins is shown in Figure 12. Amino acid sequence comparison of the neuralized homology regions (NHR) of *Drosophila* neu and human neu1, neu2 and neu3 proteins is shown in Figure 12. Amino acids that are identical in human and *Drosophila* neu proteins are highlighted in white on black background. Amino acids that are similar in human and *Drosophila* neu proteins are boxed and highlighted on grey background. h1I, NHR1 of human neu1; h2I, NHR1 of human neu2; h3, NHR of human neu3; dI, NHR1 of *Drosophila* neu; dII, NHR2 of d-neu; h1II, NHR2 of human neu1; h2II, NHR2 of human neu2.

Expression of *neu1* mRNA is highest in adult, mature neurons (Fig. 2-7). *neu2*, in contrast to *neu1*, is expressed at high levels already in the embryonic brain and the expression levels decrease during postnatal development (Fig. 13). Total RNA was isolated from the indicated mouse and rat brain regions and non-neural tissues and the levels of *neu2* transcripts were analyzed by RNase protection assays. cRNA probes that were used for detection of *neu2* transcripts covered the region encoding the NHR1 of rat *neu2*. Specific protected fragments are indicated on the left of the panel. *R-NEU2,* rat *neu2* transcript; *M-NEU2*, mouse *neu2* transcript. *E*, Embryonic day; *P*, postnatal day; *ad*, adult; *HC*, hippocampus; *CTX*, cerebral cortex; *OLF,* olfactory bulb; *STR*, striatum; *THA*, thalamus; *HTH*, hypothalamus; *COL*, colliculi; *MID*, ventral midbrain; *CBL*, cerebellum; *PONS*, pons; *MED*, medulla;*SP.C,* spinalcord; *PIT,* pituitary; *MUS,* muscle; *SPLCE*, spleen *tRNA*, yeast tRNA as a negative control.

neu3 is widely expressed, with highest levels in immune tissues spleen and thymus, and in the lung (Fig. 14). Total RNA was isolated from the indicated mouse brain regions and non-neural tissues and the levels of *neu3* transcripts were analyzed by RNase protection assays. cRNA probes that were used for detection of *neu3* transcripts covered the region encoding the NHR of mouse *neu3*. Specific protected fragments are indicated on the left of the panel. *E*, Embryonic day; *P*, postnatal day; *AD*, adult; *HC*, hippocampus; *CTX*, cerebral cortex; *OLF,* olfactory bulb; *STR*, striatum; *THA*,

thalamus; *COL*, colliculi; *MID*, midbrain; *CBL*, cerebellum; *PONS*, pons; *MED*, medulla; *LIV*, liver; *HEA*, heart; *KID*, kidney; *LUN*, lung; *MUS*, muscle; *SPL*, spleen;*TES*, testis; *THY*, thymus; *tRNA*, yeast tRNA as a negative control.

Expression of *neu4* was detected only in muscle and heart.

EXAMPLE 7

Isolation and characterization of factors interacting with Neu1

An adult rat brain library was screened using a yeast two hybrid system with a mouse Neu1 protein as a ligand with which to isolate and identify proteins that interact with Neu1. Fifty-three (53) clones were isolated. cDNAs that yielded more than 1 clone were sequenced and identified as four interactors: NeuI-1, NeuI-2, NeuI-3, and NeuI-4.

Sequence analyses showed that all interactors were novel proteins and contain RING finger domain located in the C-terminus of the protein. NeuI-1 (4 clones) is a novel splice variant (SEQ. ID. NO.: 29) of zinc finger protein Miz1/PIASX/ARIP3 (GenBank accession number NM_008602; AF077953; AF077954; AF044058). NeuI-2 (3 clones) is a fourth homologue (SEQ. ID. NO.: 30; GenBank accession number AF277171; AF302084) of zinc finger protein ZNF127 (GenBank accession number U19106; U19107), NeuI-3 (9 clones) has highest homology to a human hypothetical protein (GenBank accession number AK001459) and to a *Drosophila* hypothetical protein (AAF56052.2) produced from CG4813 gene of a genomic scaffold (GenBank accession number AE003740) (SEQ. ID. NO.: 31), and NeuI-4 (12 clones) is the homologue of the androgen receptor co-activator ARA54 (SEQ. ID. NO.: 32; GenBank accession number AF060544).

Expression of *neuI-1* - *neuI-4* mRNAs was analyzed in developing and adult rat brain and non-neural tissues (Figure 15). Total RNA was isolated from the indicated rat tissues and the levels of transcripts were analyzed by RNase protection assays with cRNA probes specific for NeuI-1, NeuI-2, NeuI-3 and NeuI-4 transcripts. Specific protected fragments are indicated on the left of each panel. Bottom panel shows the levels of GAPDH mRNA in the RNA samples. *E*, Embryonic day; *P*, postnatal day; *ad*, adult; *cbl*, cerebellum; *ctx*, cerebral cortex; *stem*, brainstem; *hc*, hippocampus; *hc + KA*,

hippocampus from rats treated for 4h with the glutamate receptor agonist kainic acid; *hea*, heart; *kid,* kidney; *mus*, skeletal muscle; *spl,* spleen; *thy*, thymus; *tes,* testis; *li*, liver; *lu*, lung; *tRNA*, yeast tRNA as a negative control.

All the identified Neu1 interactors were expressed in the brain and skeletal muscle, tissues where *neu1* mRNA is predominantly expressed. This suggests that neuI-1 - neuI-4 interact with Neu 1 protein and modify its activity *in vivo*.

EXAMPLE 8

Neu1 affects the activation of immediate early genes (IEGs)

The function of Neu1 as a calcium-dependent transcriptional repressor was studied by transfecting Neuro2A cells with *m-neu1*-CMV expression vector. This system was used to examiner whether or not Neu1 could suppress transcription of IEGs in response to the simultaneous stimulation of cells with calcium, ionophore A23187, and forskolin. As a combination of forskolin with calcium, ionophores leads to increased c-fos mRNA levels in Swiss 3T3 cells (Mehmet H, 1990). Because the induction of immediate early genes by Ca^{2+} influx requires cAMP-dependent protein kinase in PC12 cells (Ginty DD, 1991), it was decided to apply the forskolin and ionophore co-treatment to Neuro-2A cells.

Upon transient transfection of m-Neu1 into Neuro2A cells, activation of transcription of several IEGs (c-fos, junB, junD, c-jun, fra-1, and fra-2) from their endogenous promoters was significantly reduced in response to the raised intracellular Ca^{2+} (Ca ionophore) and cAMP activity (forskolin) levels. Neu1 elevated levels affected the amplitude of the IEG mRNA induction, however, had no effect on the time course of induction. This finding couples Neu1 to stimulus (calcium)-dependent transcriptional regulation.

EXAMPLE 9

Interaction with TBP

Because m-Neu1 affects a variety of target gene promoters in transient transfection assays, it was hypothesized that m-Neu1 functions by interfering directly with the function of Pol II complex, particularly suppressing the TBP transcriptional

activity. To examine this possibility, m-Neu-CMV expression constructs were cotransfected together with m-TBP-CMV. The effect of this procedure on thymidine kinase (tk) promoter was examined. Increasing amounts of Neu-CMV while mTBP-CMV amounts were kept constant (and vice versa) resulted in the decreased activity of the reporter gene in Neuro2A cells. These results suggested that m-Neu1 could repress transcription by direct interaction with TBP.

Example 10
Neoplastic Diagnostic Assay Using Genomic DNA

A biopsy is obtained from a subject possibly suffering from a neoplastic disease, such as an astrocytoma. Following excision of the sample, the tissue is immediately minced and quickly frozen in liquid nitrogen. A sample 1 gram sample of tissue is then ground with a prechilled mortar and pestle for suspension. The ground tissue is then suspended in approximately 1.2 ml of digestions buffer (100 mM NaCl, 10 mM Tris-Cl, pH 8.0, 25 mM EDTA, pH 8.0, 0.5% (w/v) SDS, and 0.1 mg/ml proteinase K, which is added fresh for each use). Samples are shaken and incubated for 12 to 18 hours at 50°C.

Following the incubation period, the samples are extracted with an equal volume of phenol/chloroform/isoamyl alcohol, to remove proteinatious material. Centrifuge the mixture for 10 minutes at 1700 x *g*. If phases do not resolve well, add another volume of digestion buffer, omitting proteinase K, and repeat centrifugation. Repeat the extraction until no thick white material appears at the interface. Transfer the top aqueous layer to a new tube.

To this tube is added ½ volume of 7.5 M ammonium acetate and 2 volumes of 100% ethanol. This mixture is centrifuged for 2 minutes at 1700 x *g*. Following the centrifugation, the resulting pellet is washed with 70% ethanol, and then air dried and will be resuspended in Tris-EDTA buffer at approximately 1 mg/ml.

To prevent shearing of high molecular weight DNA it may be advisable to remove organic solvents and salt by two dialyses against 100 volumes of Tris-EDTA buffer for more than 24 hours. If this step is performed, the pellet is not resuspended in Tris-EDTA buffer.

The purified DNA is analyzed for the presence or mutation of a wild type copy of a *neu* family gene using Southern Blotting and sequencing. The absence or mutation of such in the subject indicates the presence of a malignancy.

Example 11
Neoplastic Diagnostic Assay Using RNA Analysis

A tissue sample is obtained from a subject possibly suffering from a neoplastic disease. The biopsy is removed and cut into less than 2 gram pieces. These pieces are quick-frozen in liquid nitrogen. Twenty (20 ml) of tissue guanidinium solution is used to process 2 grams of tissue. The tissue guanidinium solution is prepared by dissolving 590.8 grams of guanidinium isothiocyanate in approximately 400 ml DEPC-treated H_20. To this is added 25 ml of 2M Tris-Cl, pH 7.5 (0.05 M final) and 20 ml of 0.5 M Na_2EDTA, pH 8.0 (0.01 M final). Stir overnight, adjust the volume to 950 ml, and filter. Finally, add 50 ml 2-ME.

Once the tissue guanidinium solution is added to the tissue, the sample is immediately ground in a tissuemizer with two or three ten second bursts. Following disruption, the solution is subjected to centrifugation for 10 minutes at 12,000 x *g* in a SS-34 rotor, at 12°C. To the supernatant is added 0.1 volumes of 20% Sarkosyl. This mixture is then subjected to heat at 65°C, for 2 minutes.

To this heated solution is added 0.1 grams of CsCl/ml of solution, which is mixed until it dissolves. The sample is next layered over 9 ml of 5.7M CsCl in silanized and autoclaved SW-28 tubes. These tubes are centrifuged overnight at 113,000 x *g* in a SW-28 rotor at 22°C.

Following the centrifugation step, the supernatant is removed and the tubes are inverted to drain. The bottom of each tube is removed and the RNA pelleted contained therein is placed in a 50-ml plastic tube. Three (3) ml of tissue resuspension buffer and allow pellet to resuspend overnight or longer at 4°C. Extract solution sequestially with 25:24:1 phenol/chloroform/isoamyl alcohol, then with 24:1 chloroform/isoamyl alcohol. Add 0.1 volume of 3 M sodium acetate, pH 5.2, and 2.5 volume of 100% ethanol, precipitate, and resuspend RNA in water. The sample is quantitated and analyzed for

the expression of the *neu* family of genes. Samples with aberent expression levels indicate the presence of neoplastic cells.

Example 12
Ex Vivo Exogenous Gene Expression

Cells are isolated from a subject to be transfected with a construct encoding Neu protein (Neu construct). The Neu construct is transfected using DEAE-dextran. The cells are seeded in 6-well tissue culture plates and are transfected with a total of 1-2μg of total DNA containing the Neu contruct. After 5 hours, 1 ml of DMEM containing 20% fetal bovine serum is added and the cells are allowed to incubate overnight.

Expression of Neu is then determined using immunoprecipitation with an antibody-agarose conjugate (8μg) plus 25μl of a 50% slurry of Protein A-agarose. Immune complexes are washed in a wash buffer (20mM Hepes, pH 7.4, 10mM $MgCl_2$, and 1mM DTT). The immune complexes are placed in a denaturing protein sample buffer that separated the antibody from any bound antigen. The protein samples are then run on SDS-polyacrylamide gel electrophoresis to detect the expression of a target Neu protein.

The foregoing description details certain embodiments of the invention. It will be appreciated, however, that no matter how detailed the foregoing appears in the text, the invention can be practised in many ways. Although this invention has been described in terms of certain preferred embodiments, other embodiments which will be apparent to those of ordinary skill in the art in view of the disclosure herein are also within the scope of this invention. As is also stated above, it should be noted that the use of particular terminology when describing certain features or aspects of the invention should not be taken to imply that the terminology is being re-defined herein to be restricted to include any specific characteristics of the features or aspects of the invention with which that terminology is associated. Accordingly, the scope of the invention should therefore be construed in accordance with the appended claims and any equivalents thereof.